THE ROLE OF EUKARYOTIC INITIATION FACTOR 4E (EIF4E) IN OSTEOSARCOMA METASTASIS

BY

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DISSERTATION

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ABSTRACT

The most significant problem for cancer patients is the dissemination of cancer cells and the formation of metastatic disease. Emblematic of the problem is the clinical progression seen in most patients with osteosarcoma, where metastasis to the lung is the most common cause of death. The primary research need in the field is to understand the biology of metastasis in osteosarcoma so as to improve outcomes for future patients. Unraveling the complexity of metastasis demands a focus on new tools, reagents, and biology in order to investigate hypotheses. Accordingly, this body of work introduces an outcome-linked human ostoesarcoma tissue microarray (new tool) used to detect and validate protein biomarkers across a variety of patients and an ex vivo pulmonary metastasis assay (new reagent) that allows real-time assessment of metastatic progression in a relevant microenvironment. Furthermore, cancer cells are believed to efficiently regulate protein translation at specific times and locations in a cell in response to changes in their environment. Preventing the dynamic regulation of these proteins (many of which have been associated with cancer/metastasis) may be an effective treatment strategy in the management of metastasis. Within the process of protein translation the abundance and activation of the mRNA cap-binding phosphoprotein, eukaryotic initiation factor 4E (eIF4E) is considered to be both rate and process limiting. We describe for the first time, the biological role of eIF4E (new biology) in metastatic osteosarcoma. We employed a comparative approach to study the biology of metastasis in osteosarcoma by using tissues and reagents from murine and human osteosarcomas. Using overexpression and knockdown techniques we modulated eIF4E expression in murine and human osteosarcoma cell lines and then evaluated the consequences at various steps within the metastatic cascade in vitro and in vivo. We found that suppression of eIF4E significantly delayed migration and reduced the number and size of

colonies that formed in soft agar. Additionally, suppression of eIF4E inhibited spontaneous pulmonary metastases. eIF4E overexpression did not change the phenotype of previously non-metastatic cells. These results suggest eIF4E may be a necessary, but not sufficient, requirement for metastasis in osteosarcoma. The goals of this research were to utilize these new tools and reagents to identify proteins and/or processes that define the metastatic phenotype of osteosarcoma and to use our newfound understanding of eIF4E in osteosarcoma metastasis to develop novel therapeutic strategies to prevent growth of metastases and improve treatment outcomes for patients.

To my parents, brother, and grandmother

and

To those affected by this disease, I hope this work contributes toward an improved understanding of osteosarcoma metastasis

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Chapter 1. Background

Osteosarcoma Metastasis

The single most significant negative prognostic factor in the treatment of cancer is the development of metastatic disease. Emblematic of the problem is the clinical progression seen in most patients with osteosarcoma. For most pediatric osteosarcoma patients, despite successful management of the primary tumor through multimodality approaches, the development of metastases, commonly to the lungs, is the cause of death. Less than 30% of patients that present with metastatic disease at the time of diagnosis will survive. Long-term outcomes for osteosarcoma patients have not improved in over 20 years (Meyers, 2009). The metastatic cascade is a complex multistage process. In order for a cancer cell to become a clinically detectable lesion, it must separate from the primary tumor, invade the surrounding tissues and basement membranes, enter and survive the circulation, arrest, extravasate, and then go on to survive, proliferate, and develop a blood supply at the secondary site. Each step is subject to a wide variety of influences such as apoptotic death or immunological response, thus at any point in the sequence the tumor cells may not survive. Again, metastasis remains a major problem in the management of osteosarcoma since the majority of mortality is associated with disseminated disease rather than the primary tumor. Opportunities to improve outcomes for patients who present with metastases and those at risk for metastatic progression require an improved understanding of tumor biology and metastasis. The primary research need in the field is to understand the biology of metastasis in osteosarcoma. The complexity of metastasis remains an enigma and therefore demands a focus on new tools and reagents in order to explore new hypotheses in the field.

Ezrin

Ezrin is a metastasis-associated protein. The genetic determinants of pulmonary metastasis in osteosarcoma were defined using a murine cDNA microarray to compare gene expression between the primary tumors of two clonally related highly metastatic (K7M2) and poorly metastatic (K12) murine models of osteosarcoma (Khanna *et al.*, 2001). The differentially expressed genes were assigned to six nonmutually exclusive metastasis-associated categories (based on literature review). Functional evaluation of the mouse models via multiple metastasis-associated assays (e.g., motility, invasion, and adherence) identified 10 genes that were considered to most likely describe the differences in the metastatic behavior of the two models. Ezrin was one of the 10 genes identified. After identifying ezrin as a gene that explained metastasis-associated differences in a mouse model, Khanna et al., (2004) further studied ezrin to determine its role in metastasis. Suppression of ezrin expression or mutation in ezrin significantly reduced metastasis in both osteosarcoma (Khanna *et al.*, 2004) and rhabdomyosarcoma (Yu *et al.*, 2004) model systems. To further demonstrate the relevance of the role of ezrin in metastasis, findings were extended to other species. High ezrin levels were associated with poor osteosarcoma prognosis in pet dogs and children (Khanna *et al.*, 2004). High ezrin levels were linked to increasing rhabdomyosarcoma grades in humans (Yu *et al.*, 2004). Collectively, these findings provide a strong argument for the metastasis-promoting function of ezrin.

Ezrin functions as a linker protein connecting the actin cytoskeleton and the plasma membrane. This cytoskeleton linkage to the cell membrane allows the cell to physically engage its microenvironment and results in a functional facilitation of many cell-signaling pathways

previously shown to be important in metastasis (Madan *et al.*, 2006; Tsukita and Yonemura, 1997). The mechanisms that may explain the ezrin-dependent effects on osteosarcoma metastasis are not well understood.

Ezrin and Translation

In an attempt to elucidate ezrin's role in metastasis, our laboratory used two fundamentally different noncandidate experiments to assess: (1) ezrin's physical and functional connections between various cell membrane proteins and the actin cytoskeleton (Clark *et al.*, 2000; Ohtani *et al.*, 2002) and (2) to define changes in gene expression following the suppression of ezrin, using stable expression of a full-length antisense ezrin in the highly metastatic K7M2 murine osteosarcoma cell line. More specifically, a proteomic approach based on affinity chromatography coupled with tandem mass spectrometry mass spectrometry (MS/MS) was utilized to identify ezrin-binding proteins, and murine cDNA microarrays were used to identify cDNAs that were differentially expressed in murine osteosarcoma cells. First we found 181 differentially expressed genes then, we applied functional assessment of these outliers and found a disproportionate number of ezrin binding proteins that had functions linked to protein translation and translation initiation. Accordingly, an emerging hypothesis from the affinity chromatography and microarray experiments was that ezrin contributes to metastasis through modulation of translation and translation initiation.

Translation and Translation Initiation in Metastasis

Translation is the second process of protein biosynthesis, which is part of the overall process of gene expression. It is necessarily preceded by transcription and has four phases: activation, initiation, elongation, and termination. In translation, messenger RNA (mRNA) which is used as a template to guide synthesis, is decoded to produce a specific polypeptide (Lehninger *et al.*, 1993). The initiation phase of translation is considered to be rate limiting in the process of translation. Initiation involves the small subunit of the ribosome binding to the 5'end of mRNA with the assistance of initiation factors (IFs) (Pain, 1996). There is now a growing body of evidence that links dysregulation of protein synthesis and malignant progression of cancer. Clemens and Bommer (1999) have discussed the many ways in which translational control is relevant to the topic of metastasis. For example, the efficiency of expression of important proteins involved in cell growth regulation, proliferation or cell death may be controlled at the translational level by changes in the activity of components of the protein synthesis machinery. Indeed, to progress through the metastatic cascade requires cooperative function of numerous proteins that facilitate invasion, survival, and angiogenesis. Although expression of these proteins may be regulated at many levels by various stimuli, translation of these metastasis-associated proteins is regulated primarily by eukaryotic initiation factor 4E (eIF4E) (Clemens and Bommer, 1999).

eIF4E

eIF4E was one of the candidates taken from the list of ezrin-associated outliers generated from the murine cDNA microarray experiments conducted in our laboratory. Indeed, eIF4E was

differentially expressed in the microarray comparison of high- versus low-ezrin/metastatic murine osteosarcoma cells. eIF4E is a 25 kDa cytosolic protein that binds to the 7-methyl guanosine cap at the 5' UTR of cellular mRNAs during translation initiation. Since eIF4E is found in much lower concentrations than other translation initiation factors, it is the rate-limiting component in translation initiation (Rhoads *et al.*, 1993; Sonenburg and Gingras, 1998). The activity of eIF4E is modulated by phosphorylation and its interaction with eIF4E-binding proteins (4E-BPs) (Flynn and Proud, 1996; Raught and Gingras, 1999). Under normal cellular conditions, eIF4E is bound by inhibitory 4E-BPs. These 4E-BPs block eIF4E from interaction with eIF4G, preventing the assembly of the eIF4F complex. Extracellular stimuli such as growth factors, hormones, mitogens, amino acids, cytokines and G-protein coupled receptor agonists induce 4E-BP phosphorylation at multiple sites (Gingras, 2001; Gingras *et al.*, 1999). Phosphorylation dislodges the inhibitory 4E-BPs from eIF4E enabling it to deliver mRNAs effectively to the eIF4F complex and facilitating translation by the eIF4F complex (Raught and Gingras, 1999).

eIF4E is an important modulator of cell growth and proliferation and is overexpressed in a number of malignancies including lymphomas, cancers of the breast, lung, head and neck, bladder, prostate, colon and rectum, esophagus, skin, and cervix (De Benedetti and Graff, 2004; Matthews-Greer J, 2005; Salehi Z, 2006; Salehi Z, 2007; Sonenburg and Gingras, 1998). eIF4E specifically enhances translation of distinct oncogenic transcripts (weak mRNAs) that are translationally repressed under normal cellular conditions. These weak mRNAs almost universally encode for growth regulatory proteins. It is likely that upregulation of these gene products enables tumorigenesis and ultimately metastatic progression. Preventing the dynamic regulation of these proteins may be an effective treatment strategy in the management of

metastasis.

Statement of Research Problem and Objectives

I am interested in defining the role of eIF4E in metastatic osteosarcoma. Cancer cells are believed to efficiently regulate protein translation at specific times and locations in a cell in response to changes in their environment. Within translation initiation the abundance and activation of the mRNA cap-binding phosphoprotein, eIF4E is considered to be both rate- and process-limiting. While eIF4E has been studied in a variety of epithelial tumors, there is little known about eIF4E in osteosarcoma. I propose to define the biological role of eIF4E in the metastatic phenotype of osteosarcoma cells (in vitro) and transplantable murine models of osteosarcoma (in vivo). Throughout these studies I will ask questions regarding eIF4E expression and activity using murine and human osteosarcoma cell lines. This understanding will facilitate the use of novel inhibitors of eIF4E and translation initiation currently under development in our laboratory and elsewhere.

Over-riding Hypothesis

The metastatic success of osteosarcoma is linked to enabled translational machinery, defined by the expression and activity of eIF4E. This hypothesis will be tested through a series of experiments some of which utilize new tools (human osteosarcoma tissue microarray (TMA)) and reagents (ex vivo pulmonary metastasis assay (PuMA)) developed in our laboratory in an attempt to help elucidate the underlying steps involved in tumor dissemination, colonization, and metastatic progression.

Specific Aim 1

Evaluation of eIF4E expression in osteosarcoma tissues. A large number of retrospectively collected human osteosarcoma patient samples and associated clinical outcomes were used to design and assemble an outcome-linked osteosarcoma TMA that will enable biomarker and target evaluation in osteosarcoma. The TMA is now available as a community resource. I have used this newly developed tool to survey eIF4E in these osteosarcoma patients by immunohistochemistry, for expression and an association with clinical outcome.

Hypotheses:

1. eIF4E is expressed in human osteosarcoma tissues.

2. Patients expressing higher-intensity staining of eIF4E will have a poorer clinical outcome (shorter disease-free intervals) than patients expressing lower staining intensities.

Specific Aim 2

Design a simple assay in which the process of metastatic progression at a secondary site (the lung) can be reproduced and studied over time. To address the unmet need for an assay that recapitulates the cellular and microenvironmental complexity of the metastatic site within a native 3-dimensional architecture, while allowing an "open window" for evaluation of metastatic progression, we developed an ex vivo pulmonary metastasis assay (PuMA). This assay allows real-time metastatic progression of GFP-expressing cancer cells in lung tissue. The PuMA successfully predicted high- and low-metastatic phenotypes of human and mouse cancer cell lines and was used for rapid screening of novel therapeutic agents at several dose and schedule

combinations.

Hypothesis:

The PuMA assay will predict the in vivo behavior of high- and low-metastatic human and mouse osteosarcoma cells.

Specific Aim 3

Define the biological role of eIF4E in the in vitro metastatic phenotype of murine and human osteosarcoma cells. Modulation of eIF4E expression in murine and human osteosarcoma cell lines, using overexpression and shRNA knock-down techniques will allow me to determine whether eIF4E is necessary and/or sufficient for the steps of the metastatic cascade in vitro.

Hypotheses:

1. eIF4E is expressed in murine and human osteosarcoma cell lines.

2. eIF4E is differentially expressed in clonally related mouse and human osteosarcoma cell lines.

3. Highly metastatic mouse and human cell lines will express higher levels of eIF4E than their clonally related low metastatic partners.

4. eIF4E overexpression in poorly metastatic mouse (K12) and human (HOS) osteosarcaoma cells will result in increased anchorage-independent growth, proliferation, motility, and migration as compared to controls.

5. eIF4E knockdown in highly metastatic human (HOS-MNNG) osteosarcaoma cells will result in decreased anchorage-independent growth, proliferation, motility, and migration as compared to controls.

Specific Aim 4

Define the biological role of eIF4E in the in vivo metastatic phenotype of osteosarcoma, using transplantation of murine and human cells to mice. Informative cell lines in which eIF4E has been appropriately modulated will be used to study the in vivo roles for eIF4E in osteosarcoma murine models.

Hypotheses:

1. Overexpression of eIF4E in poorly metastatic mouse (K12) and human (HOS) osteosarcoma cell lines will increase primary tumor growth.

2. Overexpression of eIF4E in poorly metastatic mouse (K12) and human (HOS) osteosarcoma cell lines will lead to a more aggressive metastatic phenotype in vivo.

3. Suppression of eIF4E in highly metastatic human (HOS-MNNG) osteosarcoma cells will impair in vivo metastasis.

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Chapter 2. A Connection Between Osteosarcoma Metastasis and Protein Translation: A Review

Osteosarcoma

Osteosarcoma (OS) is a rare malignant tumor of bone, arising from the malignant transformation of mesenchymal cells which have or will differentiate along the bone lineage and consequently produce osteoid (Kansara and Thomas, 2007; Malawar *et al.*, 2005). While OS can arise in any bone, the most common sites of primary tumors are the distal femur, proximal tibia, and proximal humerus. Beyond primary tumor growth in the appendicular and less commonly, the axial skeleton, a defining feature of OS biology is its high propensity for pulmonary metastasis (90% of metastases are to the lung) (Krishnan *et al.*, 2005). These defining features of OS are shared between both human and canine OS patients. This review describes the human disease, discusses cross-species comparative opportunities along with the common issue of metastasis, and introduces our interest in translation and its potential link to metastasis.

Osteosarcoma in pediatric patients

OS may occur at any age however it has a peak incidence in the second decade of life with a second smaller peak of incidence in the elderly population (after the age of 50 years) (Hayden and Hoang, 2006; Kansara and Thomas, 2007). Although rare, it is the most common pediatric bone malignancy in the United States (Gurney, 1999). Approximately 800-1000 cases of OS are diagnosed in the United States each year and about half of these patients are teenagers (Kansara and Thomas, 2007; Wang, 2005). OS occurs most frequently during the adolescent growth spurt in areas of rapid bone growth suggesting a relationship between rapid

growth/growth factors and risk of tumor formation (American Cancer Society, http://www.cancer.org/docroot/home/index.asp). OS may occur more frequently in males than females (60% versus 40%) and slightly more often in African Americans than Caucasians (Gurney, 1999).

Fifty years ago, when surgery was the only available treatment, a diagnosis of osteosarcoma was often fatal. Patients had only a 15-20% chance of cure (Dahlin and Coventry, 1967; Hayden and Hoang, 2006). Fortunately, advances in chemotherapy including the introduction of single agent and combination chemotherapy along with orthopedic surgical techniques in the 1980s and 1990s increased survival rates dramatically. Today, patients with localized disease at presentation have a 65-70% chance of 5-year relapse-free survival (Bielack *et al.*, 2002; Hayden and Hoang, 2006; Kansara and Thomas, 2007; Meyers, 2009). Remaining patients will relapse (primarily to the lung) within the first 5 years, attesting to the fact that these patients have undetectable metastatic disease at diagnosis. The prognosis for patients with OS depends mainly on whether metastases are detectable at presentation. Currently, up to 20-25% of newly diagnosed patients have detectable metastases at diagnosis (Hattinger *et al.*, 2010). Patients presenting with metastases have a poor prognosis, with long-term survival rates of 10-30% (Meyers, 2009). Other prognostic indicators for early relapse include size and location of the primary tumor, response to preoperative (neoadjuvant) chemotherapy and surgical success (Bielack *et al.*, 2002). A combined approach of surgical removal of the primary tumor and systemic chemotherapy are currently employed to treat OS. Presently, standard chemotherapy protocols are based on neoadjuvant chemotherapy followed by surgical removal of the tumor and postoperative (adjuvant) chemotherapy. The use of neoadjuvant chemotherapy has the advantage of down-staging the size of the primary tumor and as such providing a more successful

opportunity for resection as well as providing an opportunity to histologically evaluate chemotherapy-induced tumor necrosis in the resected specimen. Importantly, neoadjuvant chemotherapy also delays the urgency for surgery. The Huvos grading system is a noninvasive quantitative estimate of the percentage of tumor cell necrosis (death) observed in the tumor after surgery. Patients showing tumor necrosis in at least 90% of the resected tumor specimen are classified as good responders (Huvos score 3 or 4). Importantly, the degree of necrosis in OS following neoadjuvant chemotherapy before surgery may yield prognostic information (event-free survival) that is useful for subsequent patient treatment. Chemotherapy protocols used as a follow-up to definitive surgical resection involve multiple agents and may include methotrexate (M), adriamycin (A), doxorubicin (D), cisplatin (C), and ifosphamide (I) (Hayden and Hoang, 2006). The European and American Osteosarcoma Study (EURAMOS) group was founded in 2001. A major goal of the group is to improve survival from osteosarcoma through conducting large randomized studies. The EURAMOS I trial is designed to optimize treatment strategies for resectable OS based on histological response (Huvos score) to pre-operative chemotherapy (http://www.ctu.mrc.ac.uk/euramos). In this study, the Huvos score identifies patients as either good or poor responders. Patients who achieve a good histological response to pre-operative chemotherapy, have considerably better survival than those who have a poor response. Five-year survival for those with good response is approximately 75-80%, compared to 45-55% for those with poor response (Bielack *et al.*, 2002; Whelan *et al.*, 2000). The current treatment backbone used as first line therapy in patients includes cisplatin, doxorubicin and methotrexate. The study's objectives are: (1) examine whether the addition of ifosfamide and etoposide (IE) to post-operative chemotherapy improves event-free survival for patients with a poor histological response to pre-operative chemotherapy and (2) examine whether the addition of Interferon-∝

(IFN-∝) as maintenance therapy after post-operative chemotherapy improves event-free survival for patients with a good histological response to pre-operative chemotherapy.

Despite intensification of chemotherapy and multimodality treatments for OS, no improvements in long-term survival have been seen in over 20 years. The primary cause of death for patients continues to be the development of metastasis. Patients that present with metastatic or recurrent disease have a worse clinical outcome than patients without evident metastases at diagnosis in spite of aggressive surgical and chemotherapeutic approaches. Previous studies have shown that the overall survival rate among patients with localized OS, without detectable metastases, is approximately 60–70% (Goorin *et al.*, 1984) whereas survival rate reduces to 10–30% in patients with detectable metastatic disease (Goorin *et al.*, 1984; Han, 1981; Ward, 1994). In order for patient outcomes to improve, we must improve our understanding of the biology of metastasis.

Cross-species comparisons

Cross-species comparative opportunities to understand osteosarcoma biology and therapy are strong. Companion animals naturally develop OS and the disease is almost identical in dogs and people. The clinical presentation, biology, treatment, complications, and outcomes are similar in both dogs and humans, although human patients have better outcomes and the incidence of OS is much greater in dogs (Withrow and Wilkins, 2010). Other noteworthy differences in canine OS include, the median age of onset, which occurs in adulthood in dogs. This is different from pediatric OS where adolescents are most commonly affected. In addition, physeal plates in dogs (versus children) are closed at the age of onset of OS in the breeds of dogs that are over-represented for OS development (Fan, 2010; Withrow and Wilkins, 2010). Despite

these differences, the biology and genetics of OS are highly conserved between people and dogs (Paoloni *et al.*, 2009). Importantly, the canine OS model includes spontaneous and natural development of the primary tumor and pulmonary metastases within an immunocompetent host, thus the dog serves as a valuable comparative model for OS. A newly formed National Cancer Institute (NCI)-initiated consortium, the Comparative Oncology Trials Consortium (COTC), has integrated preclinical studies in dogs with naturally occurring OS with the drug development process. The COTC has developed a canine OS biospecimen repository. This national biospecimen repository seeks to collect cancer, normal tissues, and biofluids from 3000 dogs in an effort to coordinate the generation of biological tools for use in canine cancer that will identify agents with the greatest potential to improve outcome and prioritize those agents for early human clinical trials.

Currently, the most conventional experimental system used to study OS biology is syngeneic (genetically identical or closely related, so as to allow tissue transplant), orthotopic (in the correct anatomical site) transplantation mouse models. Conditional knockout and orthotopic, transplantable, mouse models provide an experimental system to model the genetics of human osteosarcoma. These models have proven very useful in identifying regulatory pathways in OS tumorigenesis and metastatic progression as well as, providing a valuable platform for developing novel therapeutic strategies. While the etiology of OS is unknown, it is evident that tumor suppressor genes p53 and Rb (retinoblastoma) play a role, as children with familial mutation syndromes affecting either of these genes have higher incidences of OS (Hansen, 1991). Conditional knockout mice represent an experimental system that supports endogenous, spontaneous generation of OS through knockouts of critical tumor suppressor genes. These models more closely simulate the process of primary tumorigenesis and provide an opportunity

to study early steps of the metastatic cascade. For example, genetically engineered mice (GEMs) lacking the p53 and Rb genes readily develop OS; however, while p53 loss is associated with the development of OS, the Rb gene mutation, acting synergistically with p53 is not sufficient to induce osteosarcomagenesis (Berman *et al.*, 2008; Walkley *et al.*, 2008). Orthotopic, transplantable, spontaneous metastasis mouse models offer another opportunity to study metastasis. These models provide a more accurate depiction of metastatic progression as individual tumor cells derived from the primary bone lesion must successfully establish distant metastases similar to that naturally occuring in OS in humans. A potential limitation of using a xenogeneic (derived or obtained from an organism of a different species as a tissue graft) model in which human OS cells are introduced into immunodeficient mice, rather than a murine syngeneic model system is that the xenogeneic model cannot mimic the host microenvironment and tumor cell interactions that naturally occur in humans with OS (Fan, 2010).

Metastasis

As suggested above, the single most significant negative prognostic factor in the treatment of cancer is the dissemination of cancer cells and the formation of metastatic disease rather than the primary tumor. The primary research need in the field is to understand the biology of metastasis in osteosarcoma, to improve outcomes for future patients.

There is evidence to suggest that the first stages of metastasis can be an early event (Schmidt-Kittler *et al.*, 2003) and that in many patients the metastatic process has been initiated by the time of diagnosis. Emblematic of the problem is the clinical progression seen in most patients with OS. In spite of the prevalence of pulmonary metastases in OS patients, metastasis is an extremely inefficient process. Dissemination of cancer may occur through one of three

pathways: (1) direct seeding of body cavities or surfaces, (2) lymphatic spread, and (3) hematogenous spread (Kumar *et al.*, 2005; Slauson and Cooper, 2002). Hematogenous spread is typical of solid tumors such as OS. The metastatic cascade is an extremely complex multistage process. In order for a metastatic lesion to become clinically detectable, it must successfully complete a sequential series of steps of the cascade. Within this process, each step is subject to a wide variety of influences; thus, at any point in the sequence the tumor cells may not survive. Failure to complete any step results in failure to colonize a distant site. The metastatic cascade can be divided into two phases: (1) invasion of the extracellular matrix (ECM) and (2) vascular dissemination and arrest in a distant site (Kumar *et al.*, 2005). Prior to invasion of the extracellular matrix, clonal expansion, growth, diversification, and angiogenesis must occur to produce metastatic subclones within the primary tumor (Krishnan *et al.*, 2006). These "transformed" tumor cells must then separate from the primary tumor and interact with the ECM at several stages in the metastatic cascade. In order for the neoplastic cells to breach the underlying basement membrane they must first detach themselves from one another and their surroundings. After the cells are detached, it is necessary for them to attach to matrix components (e.g., laminin, collagen, and fibronectin) so that the basement membrane can be enzymatically degraded to create passageways for tumor cell migration (Slauson and Cooper, 2002). The basement membrane is degraded by serine, cysteine, and matrix metalloproteinases (MMPs). Loss of either cell-cell or cell-matrix interaction activates caspase proteases, the hallmark of apoptosis (Frisch and Francis, 1994). Tumor cells resist detachment-induced apoptosis (anoikis) by establishing contacts with other tumor cells (homotypic interactions) or with host cells like platelets and inflammatory cells (heterotypic interactions). Both types of interactions generate intracellular signals that prevent anoikis (Grossmann, 2002; Krishnan *et al.*,

2006). Also, there can be overexpression of essential proteins by cancer cells that directly inhibit anoikis. For example, TrkB, a tyrosine kinase receptor (Douma *et al.*, 2004), the integrin pair $\alpha_2\beta_3$ (Ruoslahti and Reed, 1994), adenomatous polyposis coli (APC) gene product (Beavon, 2000), focal adhesion kinase, galectin-3, and transforming growth factor-β (Krishnan *et al.*, 2006) are reportedly involved in resistance to anoikis.

Once tumor cells have invaded the surrounding tissues and migrated through the basement membranes into the bloodstream (lymphatics or peritoneal space), they are susceptible to destruction by innate and adaptive immune defenses. In addition, destruction of intravasated tumor cells by hemodynamic forces and shearing is thought to contribute to metastatic inefficiency (Weiss, 1992). However, it has been demonstrated recently that some tumor cells in the bloodstream can arrest in capillary beds and extravasate with high efficiency (Luzzi, 1998). Neoplastic cells must strategically evade detection and destruction by the immune system and other antitumor effectors at all stages of metastatic progression (Hunter, 2004; Krishnan *et al.*, 2006; Krishnan *et al.*, 2005; Kumar *et al.*, 2005; Slauson and Cooper, 2002). Adhesion among tumor cells along with adhesion between tumor cells and platelets allow tumor cells to aggregate in clumps (tumor emboli) within the bloodstream (Kumar *et al.*, 2005). These tumor-platelet aggregates may enhance tumor cell survival and implantation. Arrest and extravasation of tumor emboli at distant sites involve adhesion to the endothelium followed by exit through the basement membrane. Involved in this process are adhesion molecules such as integrins and laminin receptors and previously mentioned proteolytic enzymes (Slauson and Cooper, 2002). At the new site, the tumor cells need to survive, proliferate, develop a vascular supply, and avoid and survive apoptotic signals and host immune responses. The site at which the circulating tumor cells leave the capillaries to form secondary deposits is defined, in part, by the anatomic

location of the primary tumor and associated venous drainage (Kumar *et al.*, 2005) However, there are many observations that suggest the natural drainage pathways do not fully explain the distribution of metastasis. Others have demonstrated that there is a tendency for the first capillary bed encountered as the primary site of metastases (Weiss, 1988). Currently, it is believed that the specific site for distant metastasis is not simply due to an anatomic location, the primary tumor, or proximity to secondary sites but rather involves interactions between tumor cells and the local microenvironment at the secondary site. One theory that attempts to explain the selectivity of certain tumor types to metastasize to specific organ sites, for example osteosarcoma to lung and bone, is the "seed and soil hypothesis" first proposed in 1889 by the surgeon Stephen Paget and then experimentally tested by Fidler in the 1980s (Hart and Fiddler, 1980; Paget, 1889). Paget hypothesized that communication between the cancer cell (seed) and the target organ (soil) via molecular interactions resulted in nonrandom selection of target organs by the cancer cells. Modulation of the tumor microenvironment (soil) by the tumor cell (seed) is a critical determinant of survival of metastatic cells (Paget, 1889). Alternatively, others have suggested that target organ tropism is based on vascular access and proximity. For example, arteries are less readily penetrated than veins due to their thicker walls, however, arterial spread may occur when tumor cells pass through the pulmonary capillary bed or when pulmonary metastases give rise to additional tumor emboli. With venous invasion, neoplastic cells follow the venous flow draining the site of the tumor; hence, the liver and lungs are most frequently involved secondarily in hematogenous dissemination (Kumar *et al.*, 2005). It is important to note however that venous drainage alone does not define the sites of metastasis. Mendoza and Khanna (2009), recently suggested in their review of cancer metastasis that a critical outcome of the seed-soil interaction is resistance to the stresses tumor cells encounter that would otherwise

impede metastatic progression. The final critical step in the development of metastasis is the generation of a new blood supply (angiogenesis). Proliferation of endothelial cells is a rigidly controlled balance between proangiogenic factors (e.g., VEGF, fibroblast growth factor, and IL-8) and their antagonists (e.g., thrombospondin-1, maspin, and angiostatin) (Krishnan *et al.*, 2006; Krishnan *et al.*, 2005; Kumar *et al.*, 2005; Slauson and Cooper, 2002). In cancer, this balance is shifted toward proangiogenic factors.

Metastasis and stress

One of the most critical determinants of a tumor cell's success in metastasis may be its ability to resist the stresses that are associated with progression through the metastatic cascade and survival at secondary sites. Stresses including low oxygen tension (hypoxia), reactive species, inflammation, nutrient deprivation, and pH can challenge the growth and progression of a tumor cell and are usually initially detrimental to the tumor cell's survival. However, it is believed that such stresses may also provide a selective pressure favoring growth of more metastatically 'fit' cells (Witz, 2006). The mammalian target of rapamycin (mTOR) is a central regulator of cell growth, proliferation, survival, and metabolism. It exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Guertin, 2007). mTORC1 is a critical mediator of the cellular response to many stresses including genotoxic, nutrient, energy, and oxygen-related stresses. mTORC1 regulates the translational machinery activity as a whole and controls the translation of a subset of mRNAs that promote cell growth and proliferation (Sengupta, 2010). Major downstream effectors of mTORC1 include regulators of protein translation initiation and cell growth, ribosomal subunit S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1). Dephosphorylation of the 4E-BPs

inhibits cap-dependent translation initiation by binding and sequestering eukaryotic initiation factor 4E (eIF4E) and preventing its assembly into the eukaryotic initiation factor 4F (eIF4F) cap-binding complex (Pause, 1994).

Translation and translation initiation in cancer/metastasis

There is now a growing body of evidence that suggests links between dysregulation of protein synthesis and malignant progression of cancer. Clemens and Bommer (1999) discuss the many ways in which translational control is relevant to the topic of cancer including how the efficient expression of important proteins involved in cell growth regulation, proliferation, or cell death may be controlled at the translational level by changes in the activity of components of the protein synthesis machinery. Also, mutations that lead to changes in the structure of individual mRNAs may alter the rates at which the proteins encoded by these mRNAs are produced. Finally, disruptions in the regulation of signaling pathways that result in a loss of control of the synthesis of growth-promoting proteins (or impair the synthesis of growth-inhibitory or pro-apoptotic proteins) may alter the balance of production of important cellular components (Clemens and Bommer, 1999).

Translation is the second process of protein biosynthesis, which is part of the overall process of gene expression. In translation, mRNA is decoded to produce a polypeptide based on the genetic code. The process converts an mRNA sequence into a chain of amino acids that form a protein (Lehninger *et al.*, 1993). The mRNA carries genetic information encoded as a ribonucleotide sequence from the chromosomes to the ribosomes. The ribonucleotides are read by translational machinery. The ribosome is a multisubunit structure containing ribosomal RNA (rRNA) and proteins (Lehninger *et al.*, 1993) and is described as the "factory" where amino

acids are assembled into proteins. There are three distinct phases of translation including initiation, elongation, and termination. In order for translation to proceed there must first be activation. In activation, the correct amino acid is joined to the correct transfer RNA (tRNA). When the tRNA has an amino acid linked to it, it is termed "charged". Once the tRNA has been charged, initiation can begin (Lehninger *et al.*, 1993; Pain, 1996). Initiation involves the small subunit of the ribosome binding to the 5' end of mRNA with the help of other proteins that assist the process (initiation factors;IFs) (Pain, 1996). Elongation takes place when the next charged tRNA in line binds to the ribosome along with GTP and an elongation factor (EF). Termination of the polypeptide happens when the A site of the ribosome faces a stop codon (Pain, 1996). Of the three phases, initiation is the most important as it is considered to be rate-limiting in the translation process. Cap-dependent translation initiation is the major translation initiation pathway in eukaryotes. All eukaryotic mRNAs present a 5' terminal nuclear modification, the cap structure (Gingras *et al.*, 1999). In translation, the cap structure marks the spot where the small ribosomal subunit (40S) is to be recruited. Important in this recruitment process is the eukaryotic initiation factor 4F (eIF4F) complex. eIF4F is a 3-subunit complex composed of eIF4E, eIF4A, and eIF4G (Gingras *et al.*, 1999; Mamane *et al.*, 2004). eIF4G is a scaffolding protein that brings together eIF4F, as it has two binding sites for eIF4A and one binding site for eIF4E, but more importantly, it bridges the mRNA cap (via eIF4E) and the 40S ribosomal subunit (via eIF3) (Gingras *et al.*, 1999; Mamane *et al.*, 2004). eIF3 is associated with the small ribosomal subunit, and plays a role in preventing the large ribosomal subunit from binding prematurely (Fig. 2.1). eIF4A is an ATP-dependent RNA helicase. Finally, eIF4E is the cap-binding protein, which is a critical node in RNA regulation impacting

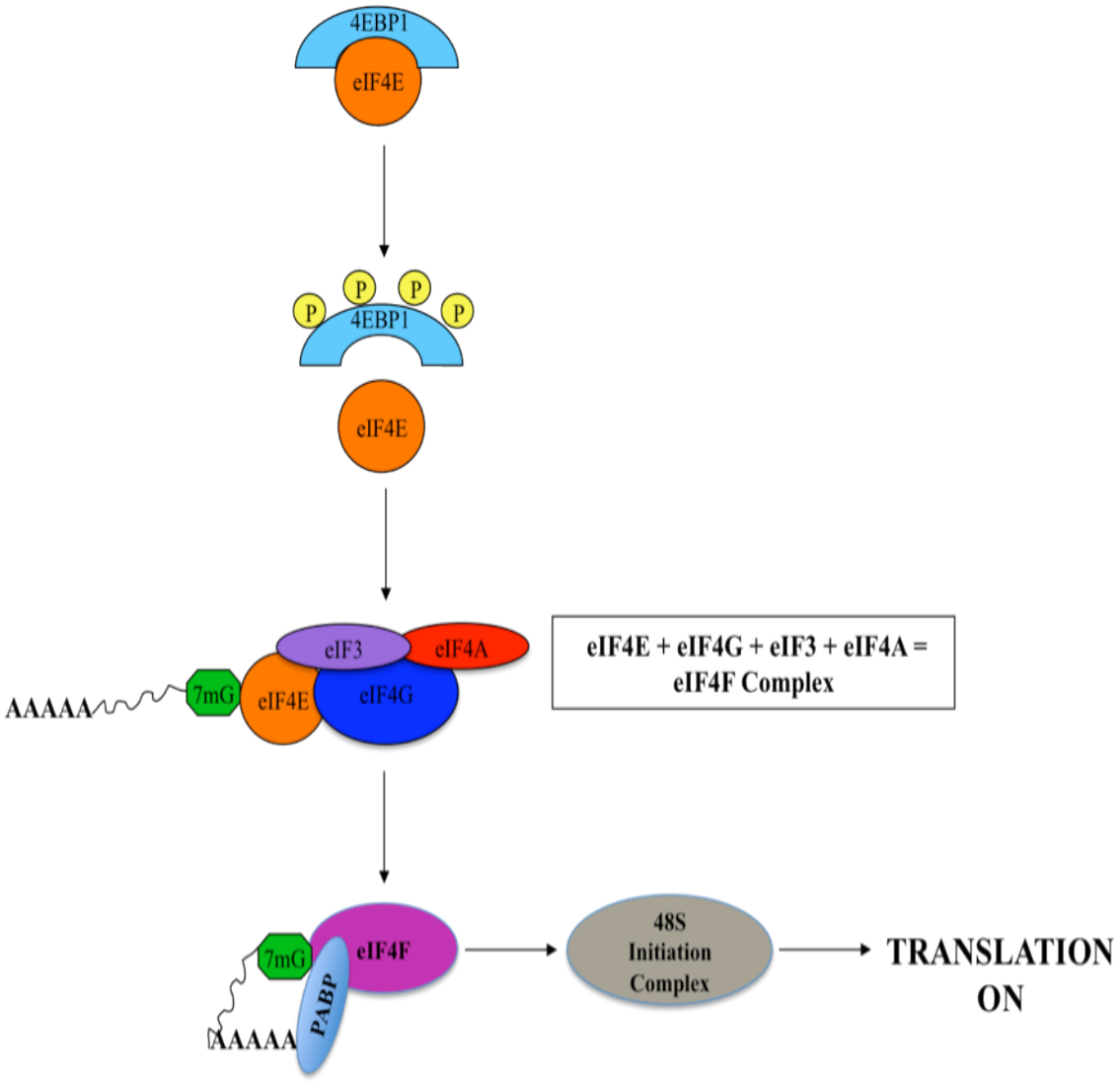

Figure 2.1. Translational Machinery. Under physiologic conditions, eIF4E is bound to its inhibitory binding protein, 4EBP1. Upon phosphorylation (P) of 4EBP1, eIF4E is liberated from 4EBP1 and able to bind with capped mRNA (7mG) and eIF4G-eIF3-eIF4A subunits, forming the eIF4F complex. eIF4E is the rate-limiting component of the eIF4F complex. eIF4F is a necessary component of the 48S initiation complex. PABP = poly(A) binding protein.

nearly every stage of cell cycle progression (Mamane *et al.*, 2004) Specifically, eIF4E coordinately promotes the mRNA export of several genes involved in the cell cycle.

A model describing a hierarchy of weakly and strongly translated proteins in cells has emerged (Baserga, 1990; Graff and Zimmer, 2003). These proteins are differentially regulated by translation. The "strongly translated" proteins are a stable set of "less regulated" proteins with short, unstructured 5' untranslated regions (UTRs) that are expressed at a basal rate with limited input from external stimuli. In contrast, "weakly translated" proteins typically have lengthy, G-C rich, highly structured 5'UTRs and are maintained as stable mRNA transcripts. These so-called weakly translated proteins are not translated unless the translational machinery is upregulated by stimuli such as growth factors or cellular stress (Bjornsti and Houghton, 2004; De Benedetti and Graff, 2004; Sonenberg and Dever, 2003). This hierarchy of proteins provides efficiency in biological systems by eliminating the need for indiscriminate, large-scale translation of all proteins at all times and locations in the cell. The model of weakly and strongly translated proteins suggests that, under specific signals and requirements, specific proteins can be translated and delivered to specialized locations in the cell (Richter and Sonenberg, 2005). For example, when the cell is exposed to stress or growth factors, there is an increased concentration of unbound (free) eIF4E available to bind with the eIF4F complex creating the specifically enhanced translation initiation that the weakly translated proteins require for efficient translation. The end result is disproportionately enhanced translation of weak proteins (De Benedetti and Graff, 2004). Interestingly, several mRNAs with complexity (lengthy, G-C rich, highly structured) in the 5' UTR (weakly translated proteins) have been associated with cancer (Table 2.1), including FGF-2 (Nathan *et al.*, 1997a), VEGF (Scott *et al.*, 1998), c-myc (Saito *et al.*,

1983), and cyclin-D1 (Stacey, 2003). Additionally, there are many proto-oncogenes that are regulated at the translational level including c-*myc*, c-*fos*, and c-*jun* (De Benedetti and Graff, 2004; Mamane *et al.*, 2004).

Table 2.1. Cap-dependent metastasis-associated mRNAs

Function	Metastasis-related gene
Cell proliferation	c-Myc CDK2 cyclin D1 ODC
Angiogenesis	VEGF FGF2 PDGF
Anti-apoptotic	Mcl-1 Bcl-2 Bcl-xL survivin
Invasion	MMP 9 heparanase

CDK2 = cyclin-dependent kinase 2, ODC = ornithine decarboxylase, VEGF = vascular endothelial growth factor, FGF2 = fibroblast growth factor 2, PDGF = platelet derived growth factor, Mcl-1 = Induced myeloid leukemia cell differentiation protein Mcl-1, Bcl-2 = B cell lymphoma 2, Bcl-xL = B lymphoma x isoform long, MMP9 = matrix metalloproteinase 9

eIF4E

Eukaryotic initiation factor 4E (eIF4E) is a 25 kDa mRNA cap-binding phosphoprotein (Rhoads *et al.*, 1993; Sonenburg and Gingras, 1998). eIF4E is an important modulator of cell growth and proliferation. It is the least abundant component of the translation initiation machinery (Rhoads *et al.*, 1993). Within translation initiation, the abundance and activation of eIF4E is considered both rate and process limiting (Rhoads *et al.*, 1993; Sonenburg and Gingras, 1998). The activity of eIF4E is modulated by phosphorylation and its interaction with eIF4E-binding proteins (4EBPs) (Flynn and Proud, 1996; Raught and Gingras, 1999). Under normal cellular conditions, eIF4E is bound by inhibitory 4EBPs. These 4EBPs block eIF4E from interaction with eIF4G, preventing the assembly of the eIF4F complex. Extracellular stimuli such as growth factors, hormones, mitogens, amino acids, cytokines and G-protein coupled receptor agonists induce 4EBP phosphorylation at multiple sites as a consequence of both the ras-ERK and PI3 kinase/AKT pathways (Gingras, 2001; Gingras *et al.*, 1999). Phosphorylation dislodges the inhibitory 4EBPs from eIF4E enabling it to deliver mRNAs effectively to the eIF4F complex and facilitating translation by the eIF4F complex (Raught and Gingras, 1999). The following mechanisms are capable of increasing levels of free eIF4E: (1) increased eIF4E expression, (2) decreased 4EBP expression, or (3) increased 4EBP phosphorylation (Huang *et al.*, 2003). It has been suggested that cancer cells have elevated free eIF4E, resulting from phosphorylation of 4EBPs, and reduced expression of the 4EBPs (Graff and Zimmer, 2003). Continual high levels of eIF4E results in disproportionately enhanced translation of weak mRNAs. Since many of these weak mRNAs encode growth regulation proteins, their upregulation may enable tumorigenesis and ultimately metastatic progression (De Benedetti and Graff, 2004; Graff and Zimmer, 2003).

Numerous studies have now implicated eIF4E in tumor formation and, potentially, metastatic progression. Overexpression of eIF4E in cell lines, NIH3T3, CREF, and MM3MG has resulted in cellular transformation and tumorigenesis (De Benedetti *et al.*, 1994; De Benedetti and Rhoads, 1990; Lazaris-Karatzas *et al.*, 1990; Li *et al.*, 2001). Antisense RNA-mediated suppression of eIF4E suppressed proliferation and changed cell morphology in HeLa cells (De Benedetti and Rhoads, 1990) and suppressed soft-agar colonization as well as tumor formation and growth in *ras*-transformed CREF cells (Rinker-Schaeffer *et al.*, 1993). Furthermore, the ability of the *ras*-transformed CREF cells to invade surrounding normal tissues and metastasize was markedly reduced as well (Graff *et al.*, 1995). Expression of antisense RNA to eIF4E in human breast, head and neck cancer cell lines suppressed tumor formation and angiogenesis (DeFatta *et al.*, 2000; Nathan *et al.*, 1997a; Nathan *et al.*, 1997b). Finally, functional blockage of eIF4E by expressing 4EBPs can revert the transformed and tumorigenic phenotype (Rousseau *et al.*, 1996). Overexpression of eIF4E has been documented in human carcinomas of the breast (De Benedetti and Graff, 2004; Kerekatte *et al.*, 1995; Scott *et al.*, 1998), head and neck (Franklin *et al.*, 1999; Nathan *et al.*, 1997b), bladder (Crew *et al.*, 2000), cervix (Matthews-Greer *et al.*, 2005), lung (Rosenwald *et al.*, 2001; Seki *et al.*, 2002), prostate (Graff, 2009), colon and rectum (Berkel *et al.*, 2001; Rosenwald *et al.*, 1999), as well as in non-Hodgkin's lymphomas (Wang *et al.*, 1999) when compared with normal tissues and benign lesions. Collectively these data suggest that eIF4E may play a key role in both tumor formation and metastatic progression by specifically enhancing the translation of a subset of key genes (weakly translated proteins) necessary for overriding normal growth constraints (c-myc, cyclin D1), inducing angiogenesis (VEGF, FGF-2), and facilitating tumor invasion and metastasis (MMP-9, heparanase) (Jiang and Muschel, 2002; Yang *et al.*, 2003; Zimmer *et al.*, 2000). eIF4E

enables cells to efficiently coordinate the translation of these needed transcripts during metastatic progression, thus increasing success in the demanding process of metastasis. While there has been a wealth of evidence in both experimental cancer models and in human cancer tissues implicating eIF4E in tumor development and progression, the majority of this work has been conducted in epithelial tumors and mouse fibroblast cells. Expression and activity of eIF4E in mesenchymal tumors, particularly osteosarcoma, have not been reported.

A clearer understanding of metastasis biology is required to improve cancer mortality, especially in osteosarcoma patients. In addition, a better understanding would allow development of novel therapeutic strategies to prevent growth of metastases. One opportunity for improved translational research is the comparative oncology approach. Cross-species comparisons in OS assist in establishing a better biological understanding of the complexity of the metastatic cascade in OS. The comparative perspective across species lines can also facilitate the discovery of novel targets for treatment. This is essential, as the development of pulmonary metastases in OS patients remains the most common cause of death. Less than 30% of patients that present with metastatic disease at the time of diagnosis will survive. Unlike most other cancers, in OS, when possible, the resection of pulmonary metastatic nodules is the first line of treatment in recurrence. Furthermore, the resistance of these pulmonary metastases to currently available therapy is common. As such, new treatments are needed. A review by Khanna (2008) summarizes novel therapeutic targets under discovery and development for OS. Advances in the basic scientific understanding of three common clinical features of the disease have uncovered new targets based on the following: (1) the origin of osteosarcoma (bone or mesenchymal cells), (2) the metastatic process, and (3) the metastatic lung lesion (Khanna, 2008). Prioritization of these targets for pre-clinical and clinical trials have shown promise for improving outcomes in

the OS patients who fail conventional treatment. A comparative perspective on the problem of OS metastasis that utilizes a cross-species approach may offer unique opportunities to assist in this prioritization and generate new hypotheses related to this important clinical problem.

We believe that opportunities to improve outcomes for patients who present with metastases and those at risk for metastatic progression require an improved understanding of tumor biology and metastasis. The primary research need in the field is to understand the biology of metastasis in OS. The complexity of metastasis remains an enigma and therefore demands a focus on new tools and reagents in order to explore new hypotheses in the field. In our laboratory we have utilized new tools (human osteosarcoma tissue microarray (TMA)) and designed new reagents (ex vivo pulmonary metastasis assay (PuMA)) in an attempt to help elucidate the underlying steps involved in tumor dissemination, colonization, and metastatic progression. To build on our understanding of metastasis biology, we designed and constructed a robust outcome-linked TMA to compliment other TMAs currently available and address the limitation of small sample size while providing a wider variety of sample types that characterize the disease including pre-treatment excisional biopsies, post-treatment definitive resections, and lung metastases. We have used our TMA to detect and validate protein biomarkers, including eIF4E, across a variety of patients. In addition, we have further developed mechanisms to view metastasis at the single cell level through the design of a simple, ex vivo, image-based, PuMA that allows real-time assessment of metastatic progression of GFP-expressing tumor cells in a relevant tumor microenvironment. We have used the PuMA to evaluate novel therapeutics that specifically target metastatic progression and metastatic lesions in OS in a timely manner. Also, the PuMA faithfully predicted high- and low-metastatic phenotypes of human and mouse cancer cell lines and the clonally related variants with greater metastatic propensity in vivo were also

associated with greater metastatic phenotype in the PuMA. These tools will advance our understanding of both OS metastasis biology and therapeutic strategies.

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Chapter 3. Evaluation of eIF4E Expression in an Osteosarcoma Specific Tissue Microarray

ABSTRACT

The ability to define osteosarcoma (OS) patients at greatest risk for metastatic progression and non-responsiveness to conventional therapy is currently not possible. Such biomarkers are needed to predict overall prognosis, probability of metastases at diagnosis, and response to chemotherapy. The tissue microarray (TMA) serves as a powerful tool for detecting and validating protein biomarkers across a variety of patients. We constructed a novel outcome-linked TMA to add to and address shortcomings of currently available osteosarcoma tissue resources. To test the utility of our TMA we surveyed the expression of eukaryotic initiation factor 4E (eIF4E) in osteosarcoma patients using immunohistochemistry. Aberrant regulation of translation initiation is a feature of many cancers. eIF4E is central to initiation of protein synthesis. Its expression and activity have been implicated in tumor formation and potentially malignant and/or metastatic progression in some carcinomas. We found that eIF4E was uniformly expressed in osteosarcoma patient samples. No association was found between eIF4E and outcome in osteosarcoma patients. This novel osteosarcoma TMA provided a facile mechanism to assess the role of a relevant protein biomarker in osteosarcoma.

INTRODUCTION

Osteosarcoma is the most common primary malignancy of bone. Approximately 400 new cases of osteosarcoma are diagnosed in pediatric patients in the United States each year (American Cancer Society, http://www.cancer.org/docroot/home/index.asp). For most pediatric osteosarcoma patients, despite successful management of the primary tumor and multi-agent adjuvant therapy, the development of metastases, commonly to the lung, is the most common cause of death. Opportunities to improve outcomes for patients who present with metastases and those at-risk for metastatic progression requires an improved understanding of tumor biology. Defining patients at the greatest risk for metastatic progression and non-responsiveness to conventional therapy is not currently possible. Such prospective identification of patients at highest risk would allow the use of novel therapeutic agents in patients at greatest need. The definition and or validation of biomarkers that predict outcome requires readily available patient samples that are linked to complete clinical follow-up. Tissue microarrays (TMAs) are well-recognized, widely used tools that enable rapid analysis of large patient cohorts for the expression of protein biomarkers using archival paraffin-embedded samples. To enable biomarker and target evaluation in osteosarcoma we report herein on the development of an outcome-linked osteosarcoma TMA available as a community resource. The tissue array is available through the National Cancer Institute (http://ttc.nci.nih.gov/forms/). As an example of the utility of the TMA, we asked if the expression of eukaryotic initiation factor 4E (eIF4E) was linked to cancer progression. eIF4E is a 25 kDa cytosolic protein that binds to the 7-methyl gaunosine cap at the 5' UTR of cellular mRNAs during translation initiation. Since eIF4E is found in much lower concentrations than other translation initiation factors, it is the rate-limiting component in translation initiation (Rhoads *et al*., 1993; Sonenburg and Gingras, 1998). eIF4E is

an important modulator of cell growth and proliferation and is overexpressed in a number of malignancies including lymphomas, cancers of the breast, lung, head and neck, bladder, prostate, colon and rectum, esophagus, skin, and cervix (De Benedetti and Graff, 2004; Matthews-Greer, 2005; Salehi, 2006; Salehi, 2007; Sonenburg and Gingras, 1998). Here, we asked (i) if eIF4E is expressed in osteosarcoma tissues and (ii) whether the expression levels of eIF4E are linked to clinical outcome in these patients.

We report the results of TMA immunohistochemical expression profiling of eIF4E in a variety of osteosarcoma sample types including pre-treatment excisional biopsies, post-treatment definitive resections, and lung metastases. We found eIF4E to be similarly expressed in all osteosarcoma sample subtypes.

MATERIALS AND METHODS

Patient Selection and Pathology

Patient records from 75 osteosarcoma patients (35 males and 40 females) linked to 89 samples (collected between 1984 and 2001) and 12 control tissues (formalin-fixed paraffin-embedded blocks) were provided by the Montefiore Hospital, Memorial Sloan Kettering Cancer Center, and Center for Cancer Research for the development of the osteosarcoma tissue array. Specimens were collected from the extremities, pelvis, and craniofacial bones. Written informed consent in accordance with the Ethics Committee and Institutional Review Board of the Memorial Sloan Kettering Cancer Center and/or Montefiore Hospital was obtained prior to tissue procurement. Using these tissues and associated clinical annotation a schema of tissue cores, arranged according to the sample type (biopsy, definitive resection, or resection of distant metastasis) was developed. Primary biopsy specimens were those taken at the time of patient

diagnosis prior to chemotherapy treatment. Definitive resection specimens were taken following neo-adjuvant chemotherapy at the time of definitive surgical treatment and metastatic specimens were collected at the time of relapse. All of the metastatic specimens included on the tissue array were taken from the lung. Diagnosis of osteosarcoma was confirmed by histologic review of hematoxylin and eosin (H&E) stained slides. For our purposes, clinical outcome was defined as overall survival of the patient. Occasionally, multiple specimens were taken from the same patient, for example, four primary biopsy patients also had definitive resection samples and two definitive resection patients had metastases at presentation. Final patient specimens included: 21 primary biopsies (19 patients), 48 definitive resections (47 patients), 20 metastases (14 patients), and 12 control tissues (Fig. 3.1).

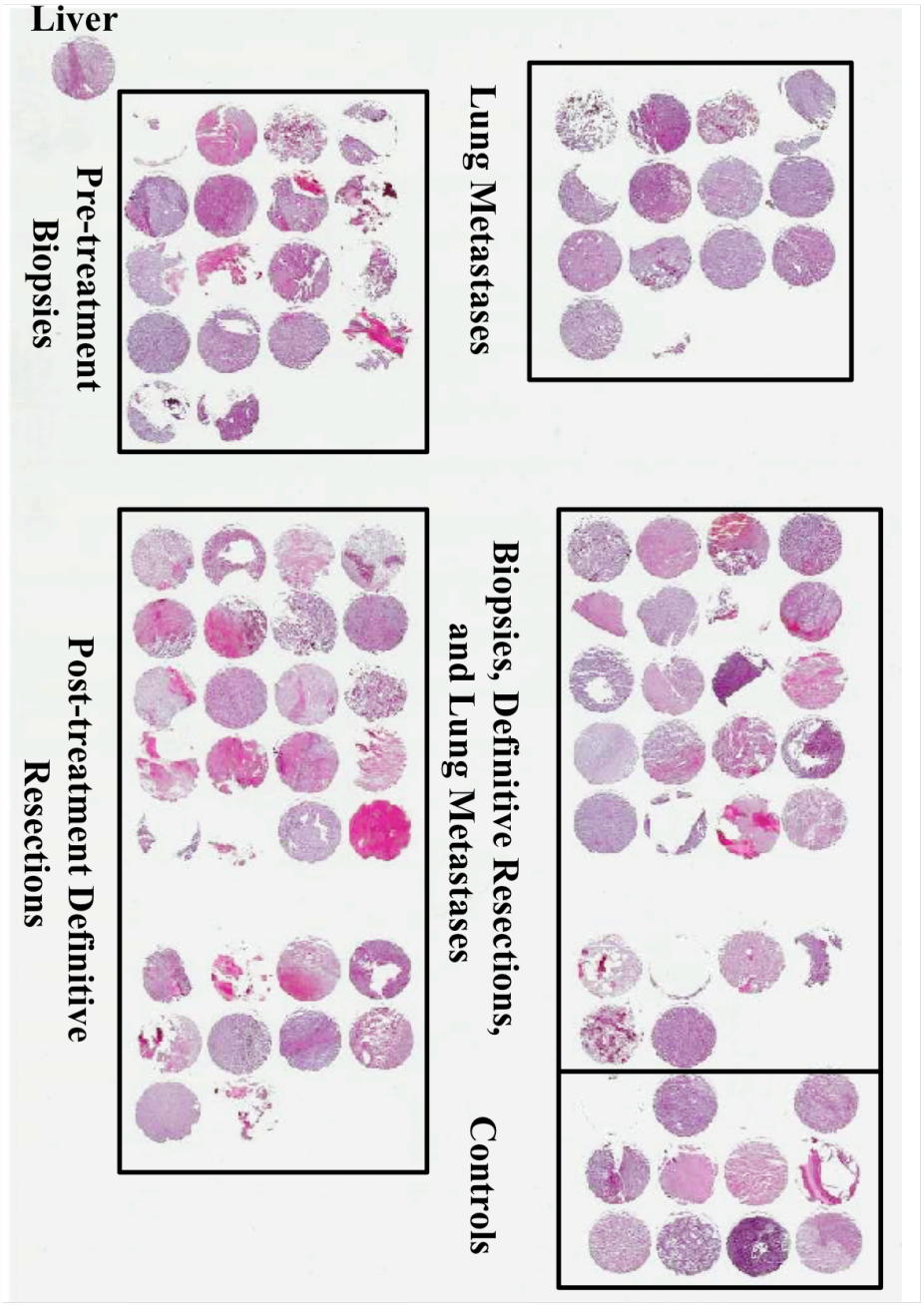

Figure 3.1. Osteosarcoma Tissue Microarray. Low magnification (0.3X) of human osteosarcoma specimens stained with H&E. Tissue microarray contains 111 tissue cores. Final patient specimens included 21 pre-treatment biopsies (19 patients), 48 post- treatment definitive resections (47 patients), 20 lung metastases (14 patients), and 12 control tissues. A liver specimen is placed in the upper left hand corner of the TMA in order to verify proper orientation.

Construction of the Osteosarcoma Tissue Microarray

The physical construction of the TMA followed the guidelines previously used by the National Cancer Institute's Tissue Array Project (http://ccr.cancer.gov/tech_initiatives/tarp/default.asp). Each individual case was represented by 1 tumor core of 1 mm that was taken from the original paraffin block. The TMA block contained 89 osteosarcoma specimens and 12 control tissues including liver, kidney, testis, muscle, lymph node, normal bone, placenta, bone cyst, fibrosarcoma, pleomorphic liposarcoma, and malignant fibrous histiocytomas. Serial 5 μm sections were cut from the TMA block and used for immunohistochemical analysis.

Immunohistochemistry

We have previously reported methods for immunohistochemical staining of TMAs (Hewitt, 2004). Briefly, primary rabbit polyclonal anti-eIF4E-antibody (Cell Signaling Technology, Danvers, MA) was applied to the TMA at a dilution of 1:100. Substitution of non-immune serum for the primary antibody was used for negative control incubations. Liver and lymph node cores were used as positive controls for eIF4E staining. Slides were developed with avidin-biotin complex kit (DAKO, Carpinteria, CA) and counterstained with hematoxylin (DAKO).

Scoring of the TMA

Before scoring, tissue cores containing less than 10% of the original tissue (3 tissue cores) or those containing less than 5% tumor cells (8 tissue cores) were excluded from the analysis. Scoring was based on staining intensity (Fig. 3.2B). The intensity of the signal was

scored as 0 (no expression), 1 (mild expression), 2 (moderate expression) or 3 (marked expression). All tissue cores were co-scored independently by two independent readers who were blinded to the clinical information. Staining intensity was based on tinctorial differences in cytoplasmic immunoreactivity. If there was disagreement about scores, the tissue cores were reviewed together and a consensus score was reached.

Clinical Outcome Measures

The clinical endpoint of this study was overall survival (Fig. 3.3A). Overall survival was defined as the time from diagnosis until death or last patient contact. Staining results were compared to overall survival (Fig. 3.3B).

Statistical Analyses

Descriptors of eIF4E staining intensities, including mean, median, confidence intervals, and log-rank tests were determined using Microsoft Excel and GraphPad Prism version 4c for Macintosh software. Log-rank and log-rank trend statistics were used for assessment of survival curves. A *P* value less than 0.05 was considered significant.

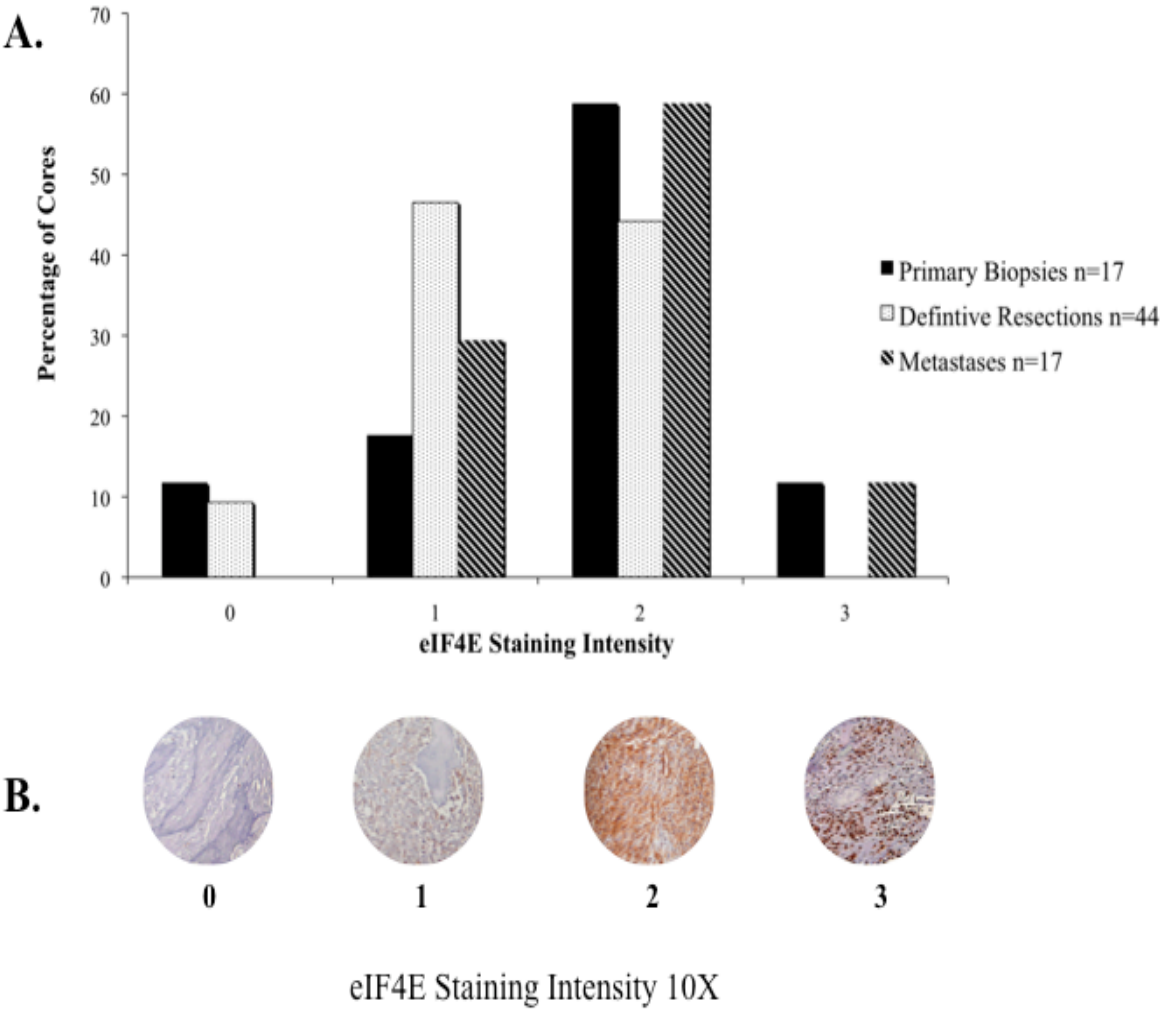

Figure 3.2. (A) Expression of eIF4E in all osteosarcoma patients. eIF4E is expressed in most osteosarcoma samples and the majority of samples have a staining intensity score of 1 or 2. **(B)** Representative tissue cores of osteosarcoma immunoreactivity for eIF4E. Scoring was based on tinctorial differences in staining intensities (i.e., 0, 1, 2, and 3) of the tissue cores.

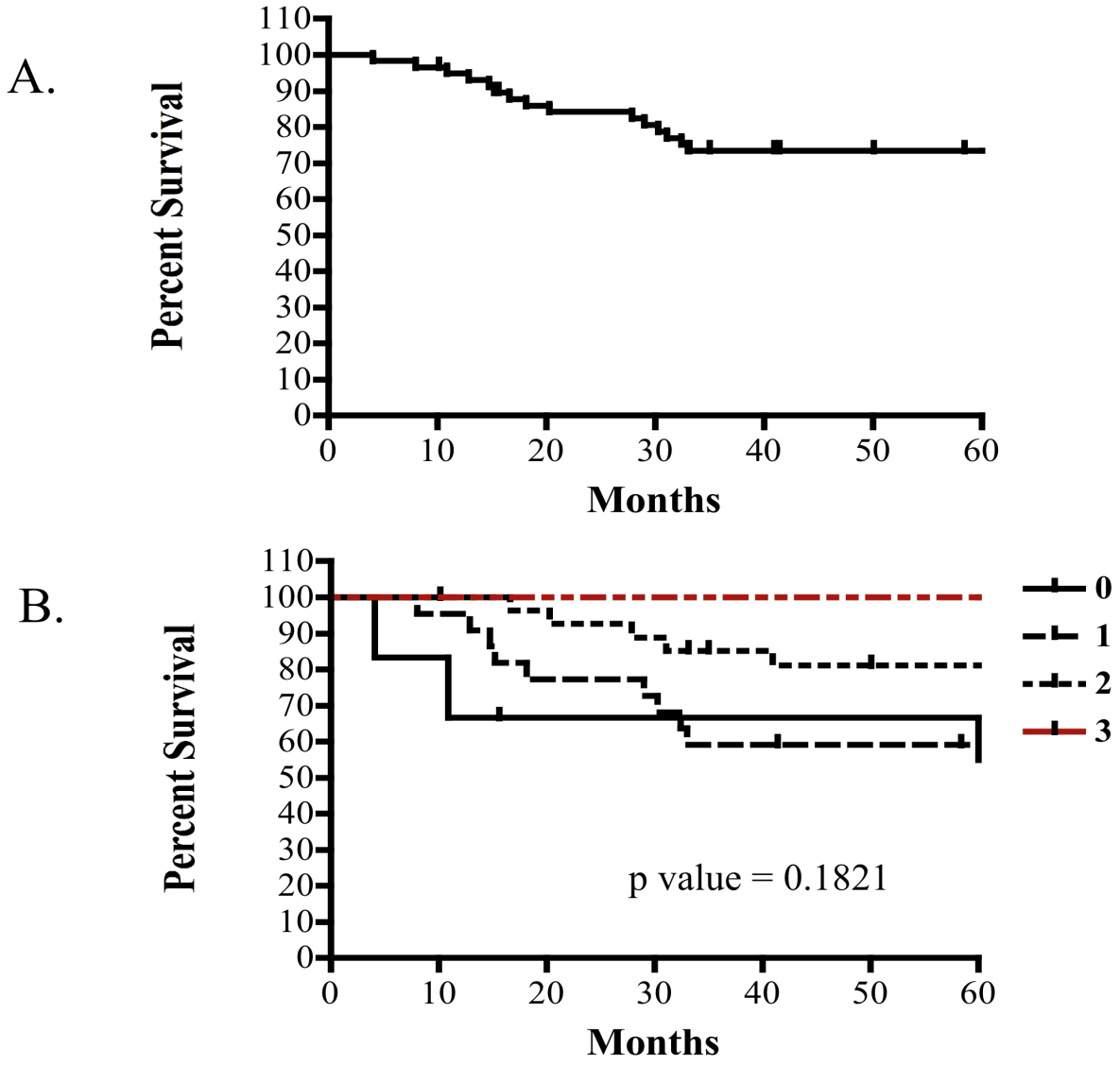

Figure 3.3. Kaplan-Meier survival curves. **(A)** Five-year survival rate for patients represented in the TMA with localized osteosarcoma without clinically detectable metastases is approximately 75%. **(B)** Kaplan-Meier survival analysis showed no significant differences in survival among distinct eIF4E staining intensities ($P = 0.1821$). Therefore, there is no correlation between eIF4E expression and overall survival in osteosarcoma patients with localized disease.

RESULTS

TMA Patient Characteristics

Sixty-seven percent of samples were taken from patients 21 years of age or less (mean-14.5, median-15). Thirty-three percent of samples included patients that were older than 21 years (mean-43.3, median-37). Specimens included tissues collected at biopsy (n=21), during definitive resection of the tumor (n=48), and during resection of distant metastasis to the lung (n=20) for a total of 89 specimens. A variety of anatomic sites and histologic subtypes (Table 3.1) were included. The majority of tumors were high-grade (47 of 48 patients; 98%) chondroblastic osteosarcomas (14 of 43 patients; 33%) that were located in the femur (20 of 52 patients; 38%). Nine patients had lung metastases at the time of diagnosis. One patient had a low-grade osteosarcoma of the metatarsus. Following procurement, samples of all specimens were decalcified, formalin-fixed, and paraffin embedded.

TABLE 3.1. Patient Characteristics

	Number of Patients
Gender	
Females	40
Males	35
Age (years)	
≤ 21	50
≥ 21	25
Sample Types	
Pre-treatment biopsies	19[a]
Post-treatment definitive resections	47[b]
Lung metastases	14
Anatomic Location	
Femur	20
Proximal tibia	11
Humerus	6
Pelvis	5
Craniofacial bones	4
Metatarsal	2
Extraskeletal	2
Ulna	1
Knee	1
Not Specified	22
Histologic Subtype	
Osteoblastic	11
Chondroblastic	14
Fibroblastic	4
Giant cell rich	4
Mixed	10
Not Specified	31

[a] Four pre-treatment biopsy patients also had post-treatment definitive resection samples

[b] Two post-treatment definitive resection patients had lung metastases at presentation

eIF4E expression and relationship with overall survival in osteosarcoma patients

To assess the utility of our TMA and test a hypothesis of interest in our studies of osteosarcoma biology, the expression of eIF4E in osteosarcoma tissues was surveyed using immunohistochemistry. Tissue controls (positive controls; liver and lymph node) exhibited diffuse cytoplasmic expression of eIF4E with staining intensities ranging from mild to marked. An assessment for eIF4E was possible in the TMA for 78 of 89 cores. Positive osteosarcoma immunoreactivity for eIF4E was detected in 71 of 78 (91%) tumors analyzed. Diffuse, mild (intensity score 1) to moderate (intensity score 2) cytoplasmic expression was observed in 67 of 78 (86%) specimens. Specifically, eIF4E was expressed in 15 of 17 (88%) primary biopsy samples, in 40 of 44 (91%) definitive resections, and in 17 of 17 (100%) metastatic lesions (Fig. 3.2A). Kaplan-Meier survival curves were used to examine differences in overall survival between each defined staining intensity (quartiles) and between high and low eIF4E expressers (Fig. 3.3B). Based on the uniform expression of eIF4E in most patient samples, it was not surprising to find that the expression intensity of eIF4E protein alone was not an independent predictor of overall survival ($P = 0.1821$) (Fig. 3.3B).

DISCUSSION

Successful management of primary tumors and advances in multimodal chemotherapy regimens have improved the overall 5-year relapse-free survival rate from 20% to approximately 60-70% for osteosarcoma patients who present with localized disease (Meyers, 2009). However, long-term outcomes for patients have not substantively improved over the last 20 years despite intensification of therapy before, during, and after the management of the primary tumor. Furthermore, the 5-year survival for patients who present with metastatic disease is 10-30% and

has remained static over this time period (Meyers, 2009). The importance of defining patients with the poorest outcomes at the time of diagnosis is critical for improvements to be seen in this disease. Unfortunately, the biological heterogeneity of this disease, coupled with the relative rarity of the cancer and limited availability of outcome-linked patient data, has made such advances difficult. TMAs are well-recognized tools that play an important role in the evaluation of biological targets in tumors. The value of TMAs in the study of rare cancers is further amplified. Currently, there are six osteosarcoma TMAs reported in the literature that are consistently used within the research community (Table 3.2) (Di Cristofano *et al*., 2010; Do *et al*., 2008; Folio *et al*., 2009; Kim *et al*., 2007; Salas *et al*., 2009; Somers *et al*., 2005). Each of these TMAs contains relatively small patient numbers, varied and limited clinical patient data, and are primarily derived from pre-treatment excisional biopsies. These small sample/patient numbers have often dampened the opportunities to assess proteins of interest in osteosarcoma and have limited the opportunity to make important associations between expressed proteins and patient outcomes. The lack of TMAs with relatively large sample sizes that contain well-documented clinical data and are derived from wide variety of sample types poses a serious challenge to physicians and researchers attempting to improve their understanding of osteosarcoma biology. The described characteristics of our array begin to address this unmet need. Although reported recently, we provide herein a complete description of the patient population as well as the patient numbers and sample types (Abdeen *et al*., 2009).

The majority of samples included in this TMA were taken from patients who had received neoadjuvant chemotherapy but still had a large percentage of viable neoplastic cells at the primary tumor site. This patient population could a potential bias in the TMA design toward patients with a poor response to chemotherapy. However, the 5-year overall survival rates for

patients included in our TMA with localized osteosarcoma (approximately 75%) is consistent with survival rates reported in the literature (Gurney, 1999; Meyers, 2008).

As an example of our TMA's utility we evaluated the expression of eIF4E in osteosarcoma tissues formatted on this array and assessed correlations between eIF4E expression, sample type, and overall survival. Our findings demonstrated that relatively uniform expression of eIF4E in both primary tumors and metastatic lesions of these osteosarcoma patients (Fig. 3.2A) and that eIF4E expression intensity was not an independent predictor of overall survival (Fig. 3.3B). It is reasonable that the uniform expression of eIF4E in osteosarcoma tissues precluded its association with outcome.

There has been a wealth of evidence in both experimental cancer models and in human cancer tissues implicating eIF4E with tumor development and progression. However, the majority of this work has been conducted in primary epithelial tumors. There is very limited information about eIF4E expression and activity in cancers of mesenchymal origin, including osteosarcoma. Because eIF4E is overexpressed in many cancers and plays a role in oncogenic transformation and tumor progression, (De Benedetti and Graff, 2004; Mamane *et al*., 2004; Proud, 2007; Ruggero, 2004) many groups are working to develop strategies to effectively target eIF4E. Novel agents in various stages of development include antisense oligonucleotides to eIF4E, RNAi or antisense RNAs that suppress eIF4E, a physical mimic of the natural ligand, suicide gene therapy, and peptide-based inhibition of eIF4E (Ko, 2009). The widespread expression of eIF4E in osteosarcoma may suggest the potential utility of these approaches in osteosarcoma.

We report herein the development of a relatively large, outcome-linked TMA for use by the community. Our TMA was built to complement other TMAs currently available and to

address the limitation of small sample size and to provide a wider variety of sample types that characterize the disease including pre-treatment excisional biopsies, post-treatment definitive resections, and lung metastases. We hope that our TMA will facilitate rapid evaluation of potential protein biomarkers and ultimately result in an improved understanding of osteosarcoma biology and therapy.

TABLE 3.2. Osteosarcoma tissue microarrays reported

TMA[a]	Samples (Patient #)[b]	Age Range (Mean)[c]	Sample Type			Grade	Huvos Score
			BX[d]	DR[e]	M[f]		
Di Cristofano	58 (50)	Unknown	22	31	5	High-40 Low-8	Unknown
Folio	46 (18) 1986-2001	6-29 (14.6)	11	0	7	High	Unknown
Kim	64 (64) 1995-2000 Males-45 Females-19	4-58 (19.4)	64	0	0	High	Unknown
Do	47 (47) 1983-2005 Males-25 Females-22	7-66 (25)	39	0	8	Unknown	I-20 II-15 III-7 IV-1
Salas	73 (73) 1993-2007	Unknown	73	0	0	Unknown	Unknown
Somers	34 (18) Males-12 Females-6	7-17 (12)	13	11	10	High	I-3 II-5 III-3 IV-0

[a] TMA = Tissue microarray

[b] Number of patients the samples are derived from

[c] Age range and mean are measured in years

[d] BX = pre-treatment biopsy

[e] DR = post-treatment definitive resection

[f] M = lung metastases

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Chapter 4. Modeling Metastasis Biology and Therapy in Real Time in the Mouse Lung[1]

ABSTRACT

Pulmonary metastasis remains the leading cause of death for cancer patients. Opportunities to improve treatment outcomes for patients require new methods to study and view the biology of metastatic progression. Here, we describe an ex vivo Pulmonary Metastasis Assay (PuMA) in which metastatic progression of GFP-expressing cancer cells, from a single cell to the formation of multicellular colonies, in the mouse lung microenvironment was assessed in real time for up to 21 days. The biological validity of this assay was confirmed by its prediction of the in vivo behavior of a variety of high- and low-metastatic human and mouse cancer cell lines and the discrimination of tumor microenvironments in the lung that were most permissive to metastasis. Using this approach, we provide what we believe to be new insights into the importance of tumor cell interactions with the stromal components of the lung microenvironment. Finally, the translational utility of this assay was demonstrated through its use in the evaluation of novel therapeutics at discrete points in time during metastatic progression. We believe that this assay system is uniquely capable of advancing our understanding of both metastasis biology and therapeutic strategies.

[1] This chapter has been previously published. A Mendoza, SH Hong, T Osborne, M Khan, K Campbell, J Briggs, A Eleswarapu, L Buquo, L Ren, S Hewitt, EH Dakir, S Garfield, R Walker, G Merlino, J Green, K Hunter, L Wakefield, C Khanna Modeling metastasis biology and therapy in real time in the mouse lung. *Journal of Clinical Investigation* 2010:120(8): 2979-2988.

INTRODUCTION

Pulmonary metastasis remains a leading cause of death for cancer patients (Eccles and Welch, 2007; Krishnan *et al.*, 2006). Opportunities to improve outcomes for these patients require a greater understanding of the biology of metastasis. In addition, there is a need to evaluate novel therapeutics, in a timely manner, which specifically target metastases and metastatic progression. Simple in vitro assay systems are not sufficient to model the complex interaction between cancer cells and the surrounding microenvironment that are necessary for metastasis (Welch, 1997). Accordingly, in vivo models of metastasis, largely in mice, have been necessary. For the most part, these models provide endpoints of metastatic outcome (i.e. yes or no metastasis) and time to late-stage metastatic events.

A temporal "black-box" exists during metastatic progression from single cells to grossly visible metastatic lesions at a secondary site. Recent attempts to shed light on this process have included imaging strategies that allow some of the steps of metastatic progression to be followed in vivo (Henriquez *et al.*, 2007). However, these approaches often involve sophisticated and expensive imaging techniques which are time-consuming and do not easily allow serial assessment of early metastatic progression at secondary sites, particularly in the lung and at the single-cell level. Challenges associated with studying metastasis have resulted in limited opportunities to include the assessment of novel treatment agents against metastatic endpoints (Khanna, 2008). Therefore, an unmet need in the field of cancer research is a simple assay in which the process of metastatic progression at a secondary site can be reproduced and studied over time.

An optimal assay would recapitulate the cellular and micro-environmental complexity of the metastatic site, within a native 3-dimensional architecture, while allowing an "open window" for

evaluation of metastatic progression. With this a goal in mind, we have developed an ex vivo Pulmonary Metastasis Assay (PuMA) in which green fluorescent protein (GFP)-expressing cancer cells proliferate and progress in lung tissue. This assay allows real-time assessment of progression from single metastatic cells to multi-cellular colonies in the lung. This assay faithfully discriminates between high- and low-metastatic phenotypes of human and murine cancer cell lines and between lung (host) microenvironments most permissive to metastasis, demonstrating the relevance and value of the approach. Finally, the assay can be easily scaled to allow for rapid screening of novel therapeutic agents at several dose and schedule combinations. Using this assay, we provide new data that support the importance of tumor cell interaction with stromal elements in the lung microenvironment as a critical determinant of the metastatic phenotype of cancer. The description and validation of this assay immediately provides researchers an opportunity to explore mechanisms for cancer progression at secondary sites and to optimally develop novel treatment approaches specific to cancer metastasis.

MATERIALS AND METHODS

Cell lines

Murine osteosarcoma K7M2 and K7M2/Ezrin antisense 1.46 (Khanna *et al.*, 2001), murine breast cancer 4T07 and 67NR (Aslakson and Miller, 1992), Met1, and DB7 (Borowsky *et al.*, 2005), human osteosarcoma HOS and HOS-MNNG (Rhim *et al.*, 1977), human breast cancer MCF7/GFP and MDA231/GFP (Barkan *et al.*, 2008), and murine Ink4a/Arf (N-Ras transformed Ink4a/Arf-null, p53-knockdown) and N-Ras transformed Ink4a-null melanoma cells (Ha *et al.*, 2007) were cultured in complete DMEM media (Invitrogen). GFP was stably expressed in all cells using pSICO-eGFP or p960-X1-685-eGFP lentiviral infection.

Lung organ culture medium

Serum-free conditions used for lung culture were first described by Siminski et al. in 1992 and were modified as follows. *Culture-medium 1*, 2X solution of M-199 (Invitrogen) was supplemented with crystalline bovine insulin 2.0 μg/ml, hydrocortisone 0.2 μg/ml (Sigma-Aldrich), retinyl acetate 0.2 μg/ml (Sigma-Aldrich), penicillin 200 U/ml and streptomycin 200 μg/ml (Invitrogen), and 7.5% sodium bicarbonate (Invitrogen). *Culture-medium 2* was used for media changes during the lung organ culture period; 1X solution of M-199 was supplemented with crystalline bovine insulin 1.0 μg/ml, hydrocortisone 0.1 μg/ml, retinyl acetate 0.1 μg/ml, penicillin 100 U/ml and streptomycin 100 μg/ml, and 7.5% sodium bicarbonate. Then a 1.2% low-melting agarose solution (FMC BioProducts) was prepared with sterile distilled water. The agarose solution was melted via microwave and kept at 40 °C before use in lung culture. The agarose solution was mixed with *Culture-medium 1* (37 °C) at a 1:1 concentration.

In vivo and ex vivo lung organ culture studies

Animal care and use was in accordance with the guidelines of the NIH Animal Care and Use Committee. The overall technique of isolated lung organ culture is shown in Figure 1. Healthy GFP-positive tumor cells (2×10^5) were delivered by tail vein injection to either female BALB/c (Taconic), SCID/Beige (Charles Rivers), FVB/N (Jackson Laboratory) AKR/J- FVB/NJ F1, or DBA/2J - FVB/NJ F1 mice. Within 15 min of tumor injection, the mice were euthanized by CO_2 inhalation. Using sterile surgical conditions in a laminar-flow hood, the mice were placed in dorsal recumbency. The sternum was removed to expose the lung. The trachea was then cannulated with a 20-gauge intravenous catheter (Terumo) and attached to a gravity perfusion

apparatus under constant 20 cm H_2O hydrostatic pressure or by syringe infusion of 1.2 ml of well-mixed c*ulture-medium 1*/agarose solution (40 °C). The trachea, lungs and heart were then carefully removed and immediately placed in a cold solution of PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 4 °C for 20 min to solidify the agarose/medium solution. Complete transverse sections (1-2 mm in thickness) were made from each lobe using a #21 scalpel blade, yielding 16-20 lung sections. Then 4 to 5 lung sections were placed on a single 1.5 x 0.7 cm sterile Gelfoam (Pfizer-Pharmacia & Upjohn Co.) section that had been pre-incubated for 2 h in 6 cm tissue culture dish with *Culture-medium 2*. Lung sections were incubated at 37 °C in humidified conditions of 5% CO_2. Fresh *Culture-medium 2* was replaced and lung tissue sections were turned over with a sterile iris thumb forceps every other day. Drug treatments were added to the culture media at either day 0 (early treatment) or day 21 (late treatment) of the culture period.

Image analysis and quantification

A LEICA-DM IRB fluorescent inverted microscope (Leica) and Retiga-EXi Fast 1394 Mono Cooled CCD camera (Qimaging) were used to capture images of GFP-positive tumor cells within the PuMA at 100 X magnification. Fluorescent events within the PuMA were acquired using OpenLab software (Improvision). Metastatic burden was quantified by measuring the fluorescent area of metastatic cells in each lung section at each time point and was expressed as either mean fluorescent area (mean fluorescent area of each lung section over 4 lung sections) or total fluorescent area (sum of fluorescent area in 4 lung sections). Fluorescent area (mean or total) was normalized to 100 pixels for day 0 to allow quantitative evaluation of metastatic progression over time.

Histology/immunohistochemistry

Formalin-fixed, paraffin-embedded mouse lung culture tissue sections were prepared at 5 µm on a transverse plane. Sections were examined using H&E and special filament stains (cytokeratin and vimentin; data not shown). Movat's pentachrome histochemical staining technique was used to determine the presence of specific connective tissue, muscular, and cellular components. Expected Movat staining results: nuclei (dark purple to black), elastic fibers (black), muscle and red blood cells (red), collagen and reticular fiber (yellow), mucosubstance (blue to blue-green) and cytoplasm (pink to brownish-red) (Movat, 1955). Ki-67 staining was performed using the LSAB®+ System-HRP kit (Dako North America, Inc) as previously described (Hsu *et al.*, 1981). Purified mouse monoclonal antibody for Ki-67 (1:25, BD Biosciences) was diluted in blocking buffer and incubated overnight at 4 ºC. Negative-control incubation was performed by substituting non-immune serum for the primary antibody. Following primary antibody incubation, slides were washed with PBS three times and incubated in biotinylated goat anti-mouse IgG secondary antibody (Dako North America, Inc) in 1:500 Dako antibody diluent for 1 h at room temperature. Streptavidin peroxidase was applied to slides for 30 min at room temperature. Color development was achieved by applying a 3-3' diaminobenzidine chromagen solution. Tissues were counterstained with hematoxylin (Sigma-Aldrich, Inc), dehydrated in a series of graded alcohols ending in xylene, mounted with coverslips and examined by light microscopy. Tissues from murine pulmonary metastatic osteosarcoma lesions were used as positive controls for Ki-67.

Transmission electron microscopy (TEM)

Mouse lung tissues were fixed in 4% formaldehyde and 2% glutaraldehyde (Tousimis) in 0.1 M cacodylate buffer (Electron Microscope Science) and processed for TEM procedures. Briefly, the tissues were washed in cacodylate buffer, post-fixed in osmium tetroxide (1% in same buffer) (Electron Microscope Science), and *en bloc* stained in uranyl acetate (0.5% in 0.1M acetate buffer pH4.2) (Electron Microscope Science). The tissues were dehydrated in a series of ethanol (e.g., 35%, 50%, 75%, 95% and 100%) followed by propylene oxide and incubated in an equal volume of propylene oxide and epoxy resin overnight. The tissues were embedded in a resin and cured in a 55 °C oven for 48 h. The thin-sectioned (75 nm) samples were mounted on a copper-meshed grid, stained in uranyl acetate (Electron Microscope Science) and lead citrate (Leica) prior to the EM examination. The digital images were taken using a H7600 microscope (Hitachi) equipped with an AMT camera (Advanced Microscopy Techniques Co.).

Comparison of metastatic progression in PuMA versus in vivo experimental metastasis

Twelve SCID mice received tail vein injection of the metastatic HOS-MNNG human osteosarcoma cell lines on day 0 of the experiment. Lungs from three of the mice were processed within the PuMA with image capture on day 0, 7, 14, and 21. Nine mice were followed in vivo. Three mice from this in vivo group were euthanized and imaged on days 7, 14, and 21 in an identical manner to the PuMA. Image analysis and quantification of metastastic progression was identical for both groups.

Statistical analyses

The statistical significance between high-metastatic and low-metastatic tumor burden was determined by unpaired *t* test with Welch's correction using Prism (version 4; GraphPad software). $P < 0.05$ was considered statistically significant.

RESULTS

Pulmonary Metastasis Assay (PuMA)

We report herein on a metastasis assay that allows real-time assessment of metastatic progression in ex vivo cultures of lung tissue (Fig. 4.1). Using the reported assay conditions, the lung architecture was maintained for over 21 days (Fig. 4.2) and provided a 3-dimensional collagen network with associated lung epithelial cells, inflammatory cells, and other stromal elements with which fluorescent metastatic cells interacted and then progressed to form metastatic colonies (Fig. 4.3 and 4.4). Routine histological examination (Fig. 4.2A), Movat pentachrome histochemical stains for connective tissue components (Fig. 4.2B), and electron microscopy (Fig. 4.2C) confirmed the maintenance of physiologically relevant collagen lung architecture over a 21-day period of ex vivo PuMA (Fig. 4.2). Confocal second harmonic generation (SHG) imaging of lung tissues confirmed the maintenance of a 3-dimensional collagen lattice in lung tissues (data not shown). Cell types present in the PuMA at early time points following lung culture included migratory cells, type I and type II pneumocytes, alveolar macrophages, vascular endothelial cells and red blood cells, airway associated epithelial cells, and stromal cells. The number and viability of some cellular populations declined through 21 days of culture (Fig. 4.2).

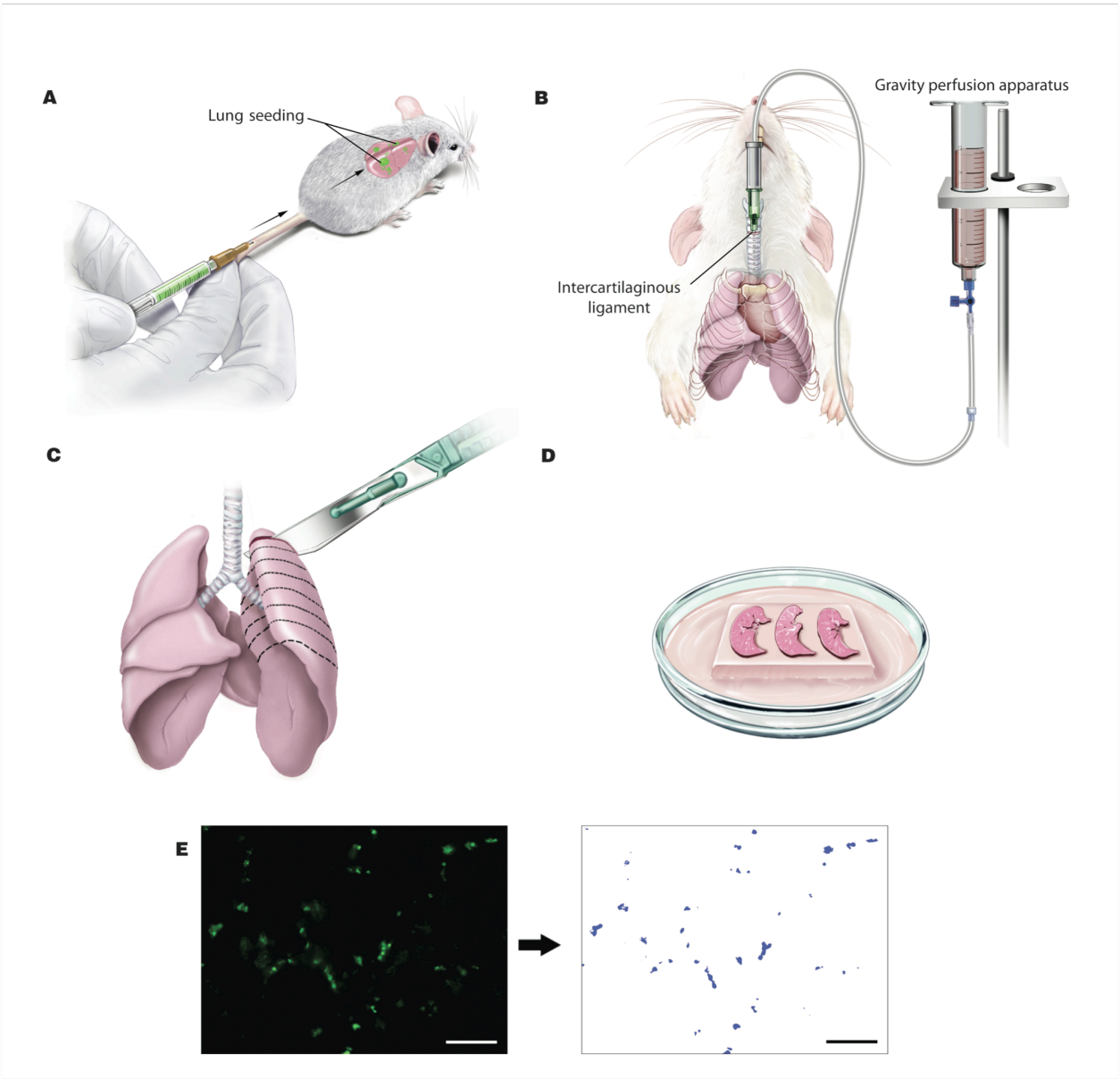

Figure 4.1. Schematic summarizing the Pulmonary Metastasis Assay (PuMA) experimental approach. **(A)** GFP-positive tumor cells (2 x 10^5) were delivered to mice by tail vein injection. **(B)** Following euthanasia, the trachea was cannulated with a 20-gauge intravenous catheter and attached to a gravity perfusion apparatus. The lungs were infused with agarose solution in the vertical position under a constant 20 cm H_2O hydrostatic pressure. **(C)** The lungs were allowed to cool at 4 °C for 20 min to solidify the agarose medium solution. Complete transverse serial sections (1-2 mm in thickness) were gently sliced from each lobe with a scalpel yielding 16-20 lung slices per lung. **(D)** 4 to 5 lung sections were placed on the sterile Gelfoam sections bathing in culture media. **(E)** Images were acquired and the area of GFP-positive cells in the each lung was quantified. Scale bar: 200 μm.

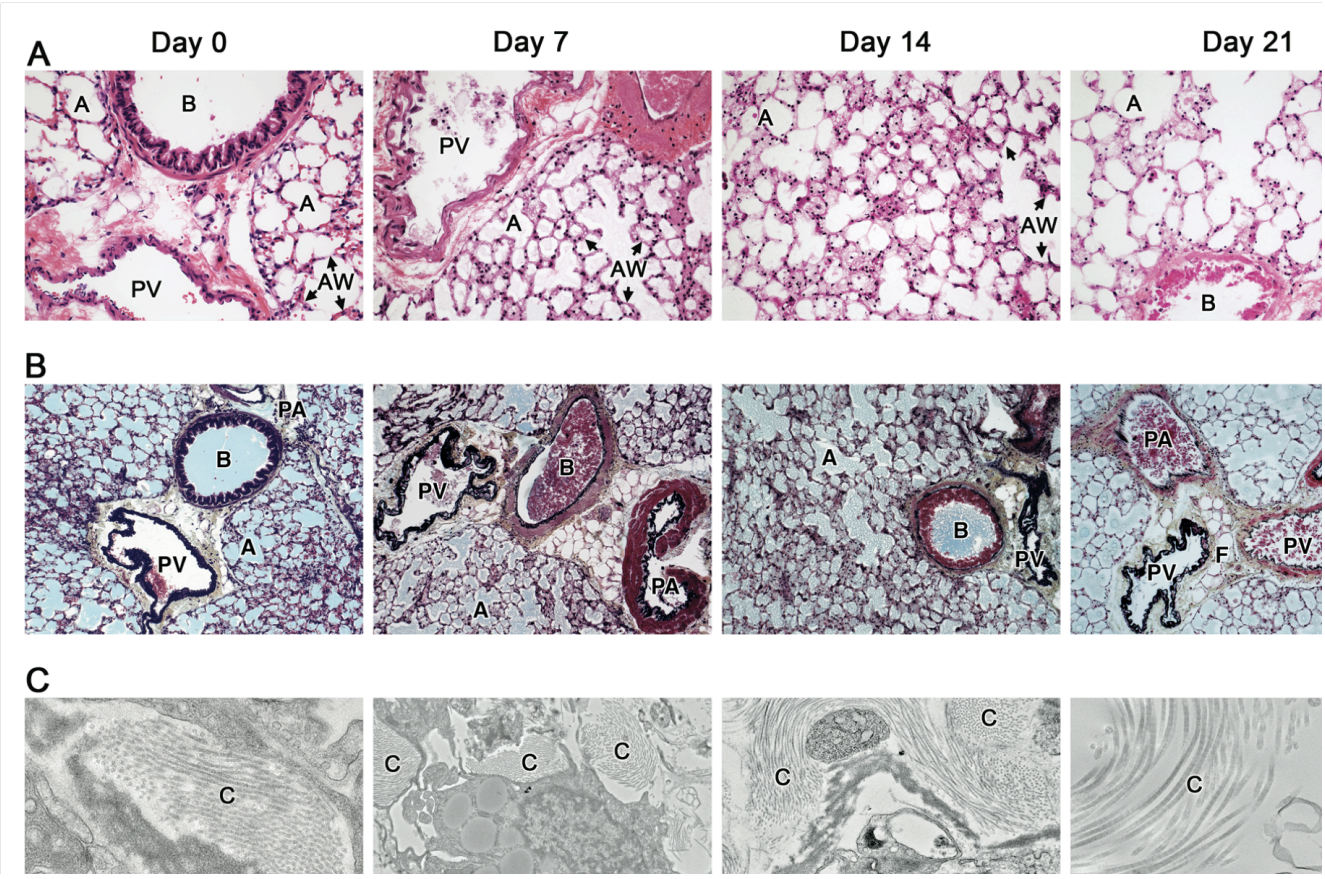

Figure 4.2 Demonstration of a viable and structurally intact pulmonary architecture in PuMA. **(A)** PuMA yields an intact lung microarchitecture. **Day 0**- Bronchioles (B) were lined by epithelia that contact the basement membrane. Alveoli (A) were uniformly expanded throughout the lung and the alveolar walls (AW with arrow heads) were normal thickness. The alveolar walls contained small numbers of migratory inflammatory cells, pneumocytes (type I and II), and endothelial cells. Blood vessels and alveolar capillaries were expanded by red blood cells (RBCs). **Day 7**- Alveoli remained expanded. There were decreased numbers of migratory cells, pneumocytes, and endothelial cells in the alveolar walls and many of those that remained contained pyknotic nuclei. **Days 14** and **21**- Alveoli, airways, and large vessels (PA – pulmonary arteries; PV – pulmonary veins) remained expanded. Alveolar capillaries and RBCs were no longer discernable, and the alveolar walls contained fewer migratory cells and pneumocytes (loss of cellularity). Overall, lung microarchitecture was remarkably unchanged. **(B)** Movat stain was used to examine the connective tissue components of the lung culture. Black elastin fibers were present in large vessels, the basement membrane supporting the airway epithelia, and within the alveolar interstitium. Black nuclei were scattered throughout the alveolar interstitium. Red muscle surrounded arteries and larger airways (B) and yellow collagen fibers were in the surrounding vascular submucosa and alveolar interstitium. Each of these components was identified at each time point. **(C)** Transmission Electron Microscopy. Stromal elements composed of collagen microfibers (C) were evident from day 0 through day 21. Scale bar: 100 μm (**A** and **B**); 1 μm (**C**).

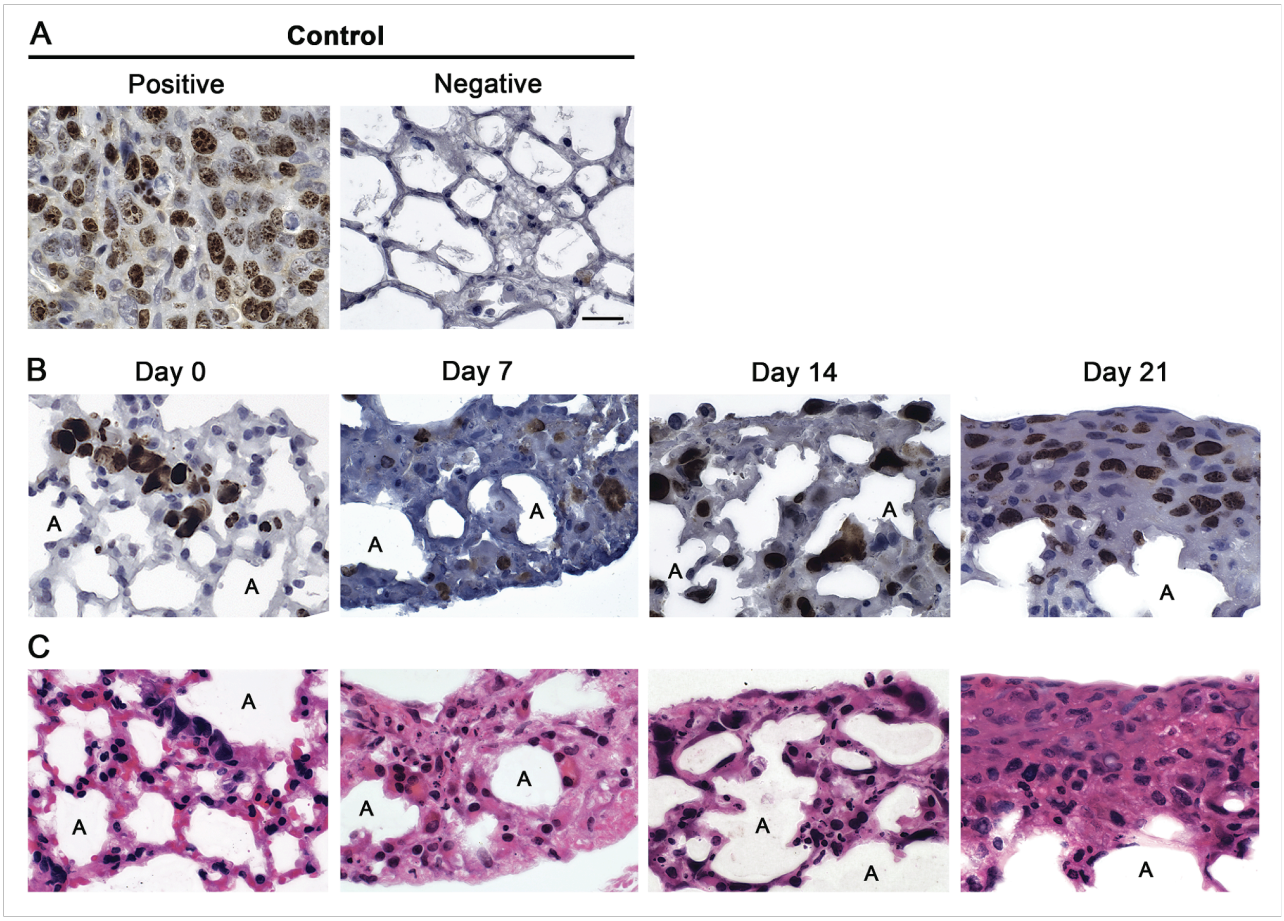

Figure 4.3. Immunohistochemical staining of highly metastatic osteosarcoma cells for Ki-67 in PuMA. **(A)** Positive and negative control, **(B)** Ki-67 immunohistochemical staining, and **(C)** H&E staining of PuMA sections confirmed proliferative competency of metastatic osteosarcoma cells, most notably at days 14 and 21. Tissue from a primary osteosarcoma lesion was used as a positive control for Ki-67. A, Alveoli. Scale bar: 100 μm.

Serial assessment of metastatic progression from single cells in PuMA

Fluorescent metastatic cells were identified microscopically and images were captured to quantify single metastatic cells and metastatic clusters using epi-fluorescence or confocal microscopy (Fig. 4.1 and 4.4). To validate the utility of the PuMA for the study of metastatic progression, we first compared previously described highly metastatic versus non-metastatic clonally related human (Rhim *et al.*, 1977) and murine (Khanna *et al.*, 2000) osteosarcoma cell lines. Osteosarcoma is a highly metastatic pediatric cancer with a high proclivity for lung metastasis. These cell lines have similar in vitro growth properties, equally form primary tumors in mice, yet show distinctive in vivo phenotypes in both experimental (i.e. tail vein injection) and spontaneous (i.e. orthotopic injection) metastasis models in mice. As shown in Figure 4.4, the number of metastatic cells that arrive in the lung with the high and low metastatic cell lines is similar at early time points. Indeed, differences in the metastatic phenotype only became evident by day 7 in PuMA with the highly metastatic cell lines forming small multicellular colonies (approximately 10-15 cells), whereas the clonally related, non-metastatic cell lines formed few multicellular clusters and then showed progressive decline in the number of single metastatic cells. The localization of single tumor cells was visualized by merging fluorescent images of metastatic cells with confocal SHG reconstruction of the collagen matrix of the lung. By day 14, large metastatic colonies were evident in the lung tissue containing highly metastatic osteosarcoma cell lines whereas very few cells could be identified in lung tissue containing low metastatic cell lines (Fig. 4.4, A-D). Interestingly, similar to our observations from in vivo studies of metastasis in mice, most metastatic colonies were observed at the periphery of the lung sections.

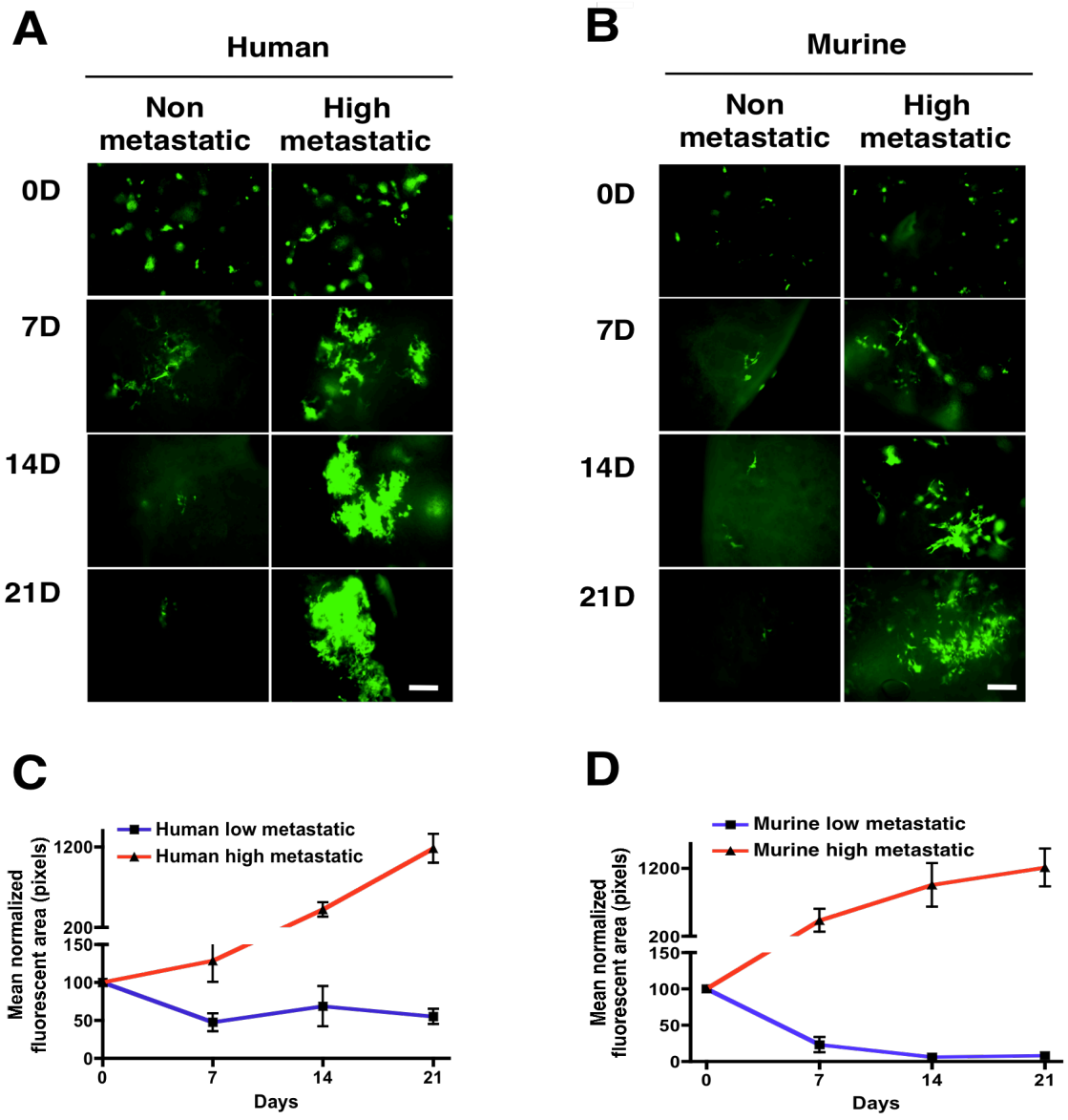

Figure 4.4. The PuMA distinguishes the phenotype of metastatic from non-metastatic cell lines. (**A** and **B**) Serial imaging of fluorescently labeled high and low metastatic human and murine osteosarcoma cells. Similar numbers of cells were seen at day 0; however, a difference between high and low metastatic cell lines was seen at day 7. Representative fields from lung are shown. Scale bar: 200 μm. (**C** and **D**) Quantification of metastatic burden (mean normalized fluorescent area) from **A** and **B** reveals a significantly lower sum of GFP positive cell area in the lung section following injection of low metastatic human and murine osteosarcoma cells. Similar results were generated using high and low metastatic pairs of human and murine breast cancer cells and murine melanoma cells (See Table 4.1). Quantification of metastatic progression in PuMA was validated using both mean fluorescent area and enumeration of surface metastatic colony count.

In order to demonstrate that the metastatic tumor cells were actively proliferating in the lung cultures, Ki-67 immunohistochemical staining was conducted during the culture period (Fig. 4.3). Indeed, positive Ki-67 staining was observed in metastatic tumor cells in the PuMA. This included single metastatic cells early after their arrival in the lung and in larger metastatic colonies at later time points. In addition, Ki-67 immunoreactivity was observed in the alveolar interstitium demonstrating the viability of alveolar interstitial cells in this assay. As further validation that metastatic tumor cell numbers increased over time in PuMA, quantitative real-time PCR for the eGFP transgene was performed on genomic DNA isolated from lung slices containing metastatic tumor cells at multiple time points. As expected, the eGFP DNA copy number increased over time, indicative of increased tumor cell number and consistent with fluorescent evaluation showing metastatic progression.

To further validate the utility of the assay in the study of metastasis biology we examined a larger series of clonally related cancer cell lines with known differences in metastatic phenotype (Summary in Table 1) (Aslakson and Miller, 1992; Barkan *et al.*, 2008; Borowsky *et al.*, 2005; Ha *et al.*, 2007; Khanna *et al.*, 2001; Lifsted *et al.*, 1998; Rhim *et al.*, 1977). The kinetics of metastatic progression in the PuMA were distinct for each cancer cell line assessed (Fig. 4.4). Nonetheless, in each case, the clonally related variants with greater propensity for metastasis *in vivo* were also associated with greater metastatic phenotype in the PuMA. It is important to note that metastatic phenotypes of these cell line pairs are indistinguishable in conventional 2-dimensional culture conditions (Table 1).

A direct comparison of the PuMA with in vivo experimental metastasis using identical cell lines and assessment points suggested that the kinetics of metastatic progression in the PuMA

and in vivo are similar (Fig. 4.5). The primary difference between PuMA and in vivo experimental metastasis was the duration of the initial period of metastatic inefficiency, during which the number of single metastatic cells detected in the lung was reduced. In the in vivo experimental metastasis setting, this period of inefficiency for osteosarcoma cells was most notable at day 7. In the PuMA, the metastatic inefficiency for these osteosarcoma cells was most notable at day 4 and had begun to recover at day 7. Similar and more extreme patterns of metastatic inefficiency were seen in both PuMA and the in vivo setting using non-metastatic cells (data not shown). Beyond the calibration between PuMA and the in vivo settings, these data suggest that a primary determinant of early metastatic inefficiency is survival of metastatic cells in the lung and is not merely a function of blood flow "washing away" metastatic cells from the lung.

Table 4.1. Descriptive summary of concordance between PuMA and in vivo studies of metastasis biology

Metastasis biology	PuMA metastatic phenotype is discernible[A]	In vivo metastatic phenotype is discernible[B]	In vitro metastatic phenotype is discernible[C]
Human osteosarcoma	Yes	Yes	No
Murine osteosarcoma	Yes	Yes	No
Human breast cancer	Yes	Yes	No
Murine breast cancer	Yes	Yes	No
Murine melanoma	Yes	Yes	No
Murine host microenvironments	Yes	Yes	No

[A] Discernible: can a blinded investigator distinguish metastatic and non-metastatic cells in the PuMA assay? Refer to Figure 4.4 for actual experimental data.
[B] Discernable: can a blinded investigator distinguish metastatic and non-metastatic cells in the in vivo studies?
[C] Discernable: can a blinded investigator distinguish metastatic and non-metastatic cells in conventional in vitro tissue culture conditions?

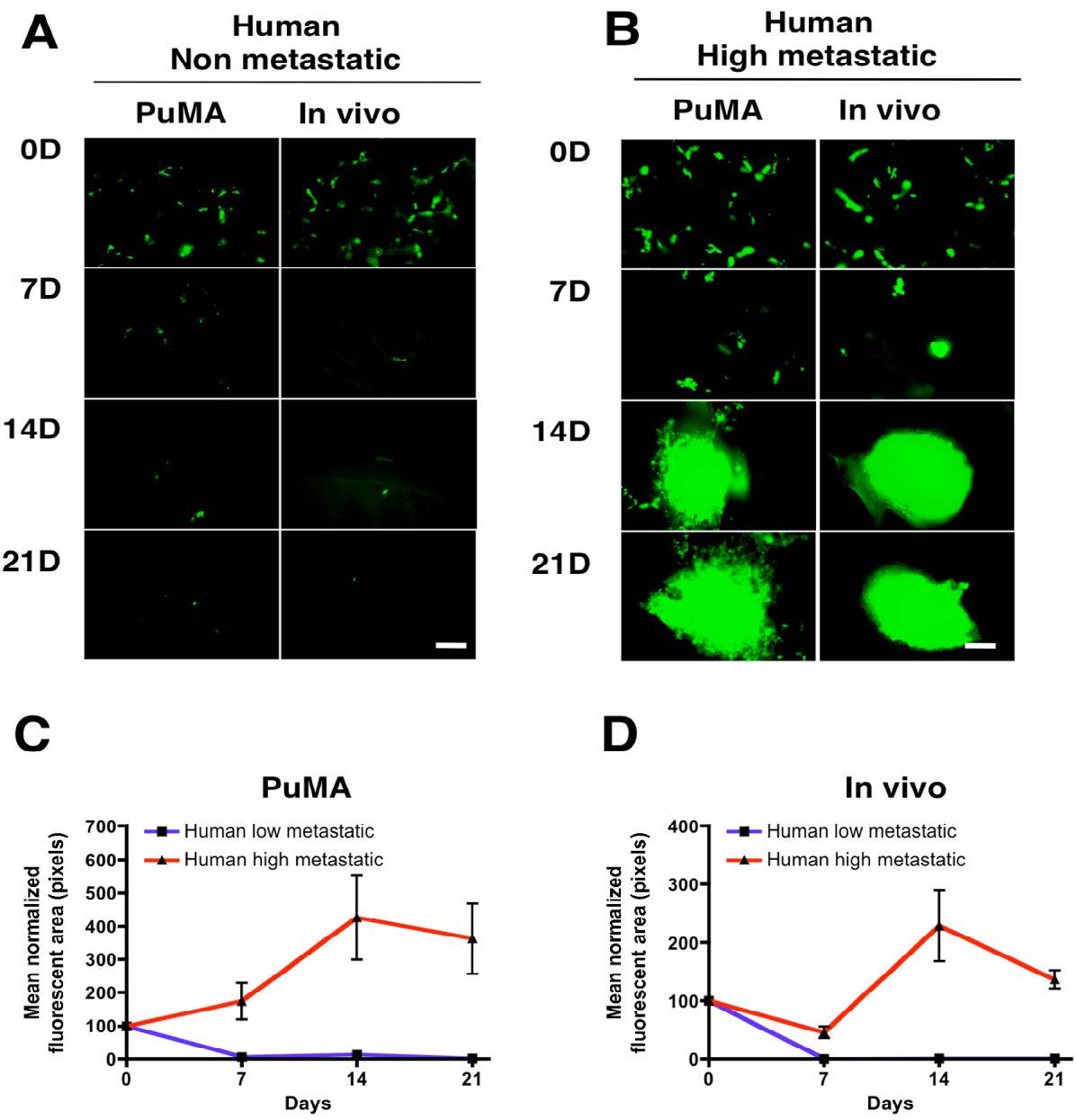

Figure 4.5. Similarities in metastatic progression in vivo compared to PuMA. A direct comparison of the metastatic phenotype of human osteosarcoma cell lines in vivo (experimental metastasis) and in the PuMA was conducted. (**A** and **B**) Serial imaging of fluorescently labeled high and low metastatic human osteosarcoma cells was conducted in the PUMA. At the identical time points as in the PuMA, lungs from mice that had received tail vein injection of tumor cells were collected and imaged. Patterns of pulmonary metastatic progression were similar in both in vivo and PuMA. Representative fields from lung are shown. Scale bar: 200 μm. (**C** and **D**) Quantification of metastatic burden (mean normalized fluorescent area) from **A** and **B**. Identical results demonstrating the similarities in pulmonary metastatic progression for murine osteosarcoma cells were seen in vivo and in the PuMA.

Since the PuMA includes cellular and stromal elements of the host, we next asked if this novel system might also allow microenvironmental (i.e. host) influences on metastatic progression to be assessed. AKR/J and DBA/2J represent murine genetic backgrounds in which primary tumor initiation and growth kinetics are identical, but where there is a 20-fold difference in metastatic propensity (Lifsted *et al.*, 1998). As predicted by in vivo studies, a more aggressive pattern of metastatic progression was seen for mammary cancer cells in the AKR/J as compared to DBA/2J mouse lung sections (Fig. 4.6 and Table 1). These results support the hypothesis that metastasis-associated differences in genetic background that have been previously reported are indeed the result of differences in the local tumor microenvironment (i.e. lung microenvironment). Furthermore, given the absence of a systemic immune component in the PuMA, these data suggest the importance of stromal elements of the extracellular matrix in the lung as primary determinants of the role of the tumor microenvironment on metastatic progression. These data further underscore the opportunity to evaluate both tumor and microenvironmental influences linked to metastatic progression within the PuMA.

Screening of therapeutic agents with potential activity against metastatic progression and metastases

Finally, we sought to determine if the PuMA could be used to identify anti-metastatic agents. The ability of therapeutic agents to diffuse through the culture media into the gelfoam material and into the lung section tissues was tested within the PuMA using tumor cells that expressed GFP under the control of a doxycycline-sensitive promoter. Within 24 h of addition of doxycycline to the culture media of the PuMA, we observed expression of GFP in tumor cells.

We then compared the activity of therapeutic agents in PuMA in relation to their activity in conventional transplantable murine models (summarized in Table 4.2). Evaluated therapeutic agents included conventional cytotoxic chemotherapy (treosulfan) and the prototypic mTOR pathway inhibitor (rapamycin). Consistent with in vivo results (Wan *et al.*, 2005), rapamycin exposures to the PuMA significantly inhibited metastatic progression at 1 μM. The anticancer activity of Treosulfan, a non-targeted cytotoxic chemotherapy, has been shown in vitro and in vivo against a variety of pediatric sarcoma cell lines (Werner *et al.*, 2008). Significant activity of these agents was confirmed in the PuMA following their addition early or late during the course of metastatic progression. The throughput and flexibility of the PuMA allowed several doses of each agent to be assessed against metastatic progression in a short period of time. The real-time observation provided by the assay allowed the assessment of each agent against either the entire metastatic progression, from single metastatic cells to metastatic clusters, or at discrete points in the course of metastatic progression in the lung.

Table 4.2. Descriptive summary of the use of PuMA in translational evaluation of novel anticancer agents with activity against metastasis

Metastasis therapy	Anticancer activity in PuMA	Antimetastatic activity in vivo	Anticancer activity in vitro
Rapamycin (14)	Yes	Yes	Yes
Treosulfan (15)	Yes	Yes	Yes

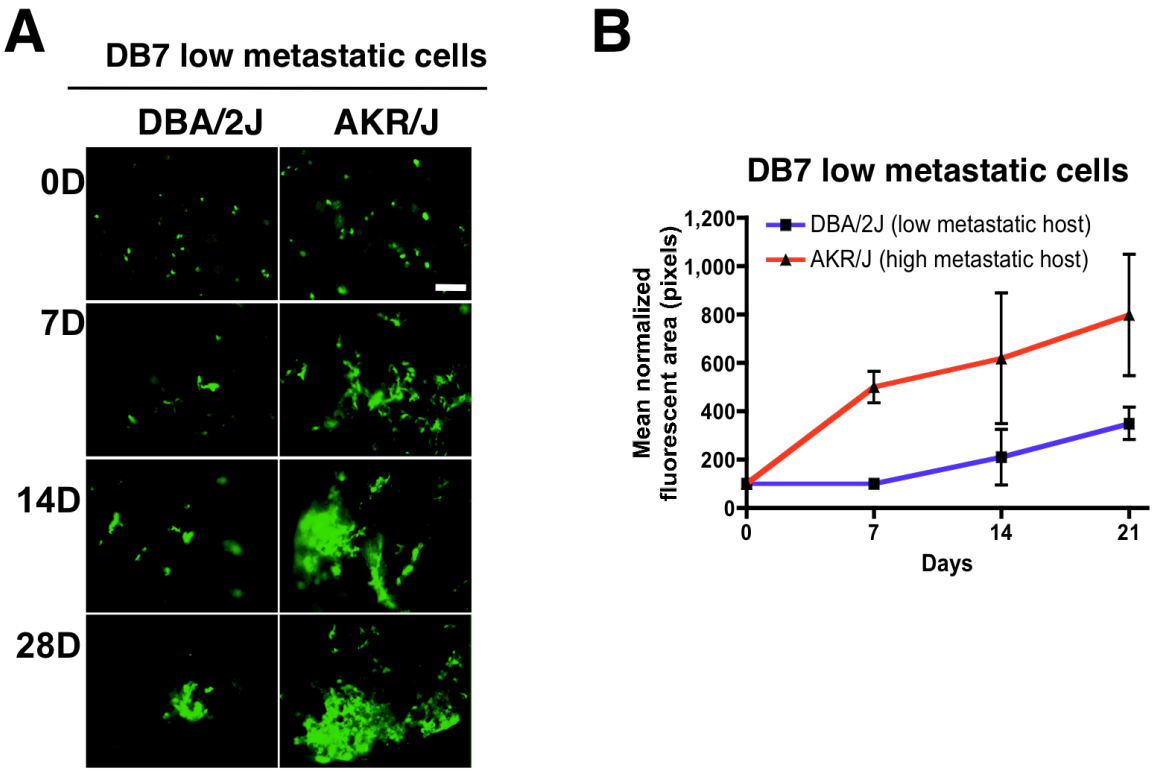

Figure 4.6. The PuMA can distinguish host microenvironments that are permissive. **(A)** Serial imaging of fluorescently labeled low metastatic murine breast cells (DB7) in high (AKR/J) and low (DBA/2J) metastatic murine host microenvironments. Similar numbers of metastatic cells are seen at day 0 in both lung environments; however, a difference in metastatic progression between high and low metastatic microenvironments can be seen at day 7. Representative fields from lung are shown. Scale bar: 200 μm. **(B)** Reduced metastatic burden (quantified by total normalized fluorescent lung area) was seen in the low metastatic phenotype of DAB/2J lung sections. Total metastatic burden in each lung section was normalized to day 0 and followed over time.

DISCUSSION

The PuMA is an ex vivo, closed system and sterile assay that allows assessment of metastatic progression from single cells to metastatic clusters up to and beyond a 21-day observation period. During this time, metastatic tumor cells interact with the 3-dimensional collagen network within the lung (the primary constituent of the lung architecture) and associated lung epithelial cells, inflammatory cells, and other stromal elements. Fluorescent metastatic cells and metastatic lesions were quantified for number, size and localization during this period. The relevance of the PuMA was confirmed by its ability to accurately define the metastatic phenotype of tumor cell lines with previously established high versus low metastatic behaviors in vivo. These predictions are noteworthy since the metastatic phenotype of these cells is not distinguished by growth in conventional in vitro culture conditions. Given the recognized importance of the tumor-microenvironment interactions for metastatic progression, it was particularly revealing to see that the PuMA was used effectively to compare the influences of distinct host microenvironments on the progression of identical tumor cells. These new data suggest the importance of the local microenvironment rather than systemic response of the host on the metastatic phenotype. Finally, the opportunity to use this PuMA approach as a translational drug development tool was then supported by the evaluation of both cytotoxic and molecularly targeted therapeutics against metastatic progression. Importantly, the flexibility of the assay allowed rapid assessment of multiple drug doses, exposure times and schedules. The PuMA fills an unmet need in the study of metastasis biology and therapy by allowing the real-time assessment of metastatic progression in a relevant tumor microenvironment.

A critical component of the assay conditions reported herein was the insufflation of 0.6% agarose to the lung (Cifone and Fidler, 1980). Without agarose insufflation the lung structure

was completely lost within 24 h. The influence of agarose density on metastatic tumor cell growth has been previously assessed (Cifone and Fidler, 1980). Conventional microscopy, immunohistochemistry, electron microscopy and second harmonic generation microscopy demonstrated the collagenous nature of the lung architecture. Based on confocal fluorescent microscopy and second harmonic generation imaging (which images the collagen matrix of the lung), we hypothesize that the 3-dimensional collagen lattice that is retained in the PuMA is a critical scaffold on which tumor cells interact with other cells of the lung microenvironment. We and others have hypothesized that the survival of tumor cells at distant secondary sites is stressful and that this stress is a primary contribution to the inefficiency of the metastatic process (Chambers *et al.*, 1995; Khanna *et al.*, 2001). Based on the results of the PuMA it is reasonable to argue that metastatic cells have an enhanced capability to engage the local lung microenvironment and manage growth on the scaffolding of the lung collagen architecture than non-metastatic cells. Indeed, survival in this 3-dimensional context may be the measurement of metastatic competency assessed in this assay. Our studies of differences in metastatic progression in the PuMA that result only from changes in the strain of mice used, now provide additional information on the importance of the interaction between tumor and tumor-microenvironment as a critical determinant of (strain/host-dependent) metastatic success. These data in the PuMA provide a unique perspective on the importance of the non-cellular stromal elements of the lung microenvironment in the progression of lung metastasis. It is recognized that different mouse strains express different ECM components. These include stromal elements observed within the microarray prognosis signatures for metastatic cancers. Based on the PuMA data, we suggest that tumor cells are responding differently to the subtle variations of ECM composition that either induce or suppress growth in the secondary site. These data are

concordant with recent reports on the role of lung collagen in regulating the dormancy phenotype of some metastatic cancers (Barkan *et al.*, 2008).

The PuMA provides an opportunity to visualize and monitor the progression of metastasis in the lung in ways not previously possible. For example, using existing in vivo mouse models we are unable to easily assess the full range of steps and cellular processes involved in metastatic progression in the lung despite the application of sophisticated imaging approaches (Cameron *et al.*, 2000). Furthermore, the PuMA complements recent assay approaches used to monitor metastatic progression, such as intravital videomicroscopy (IVVM) (Al-Mehdi *et al.*, 2000; MacDonald and Chambers, 2006). Unlike PuMA, IVVM allows the study of metastasis in the context of living tissue with blood flow. IVVM is constrained by a relatively short window of observation, most commonly restricted to hours after cellular arrival at secondary sites. Other effective approaches to image metastatic progression in the lung can be confounded by the impact of surgery needed to expose the lung and the influences of respiring or ventilated lung on image quality. In addition, several simple 3-dimensional in vitro culture systems have been used to study the metastatic phenotype of cells. However, these approaches fail to model the complexity of tumor cell-tumor microenvironment interactions necessary for metastasis (Themistocleous *et al.*, 2004). Our current description of the PuMA addresses many of the shortcomings of currently available methods to study metastasis biology. Indeed a direct comparison of PuMA with in vivo experimental metastasis demonstrated the similarity in metastatic progression seen in both ex vivo and in vivo conditions. It is reasonable, and our expectation, that the PuMA will provide opportunities for several future investigations of metastasis biology including determinants of the metastatic tumor cell, the tumor microenvironment and their interaction.

Finally, the PuMA provides a novel approach to specifically evaluate the efficacy of anti-cancer agents in the context of metastatic progression at a secondary site. Despite the fact that the most common cause of death in cancer patients is metastasis, current preclinical drug development models do not prioritize metastasis endpoints in their evaluation of new drug candidates. This is due, in part, to the inability to model metastatic progression or metastatic lesions outside the mouse. It is also due to the fact that in vitro assays of specific metastatic processes (i.e. motility or invasion) are, alone, not always informative. Although not a high-throughput option, the PuMA provides an efficient assessment of several drug/dose/schedule combinations over time. Furthermore, the PuMA allows the evaluation of a new drug candidate against either single metastatic cells or advanced metastatic lesions. In this way, the PuMA may be useful in identifying or prioritizing novel cancer therapy agents for use against metastatic progression or metastatic lesions and should therefore accelerate the development of new treatments for patients with metastasis.

Although not investigated herein, the PuMA will provide an opportunity to study later points in metastatic progression, i.e., from metastatic colony formation to development of gross metastatic lesions. This may be accomplished by delaying the initiation of the PuMA for days or weeks after the injection of tumor cells into mice. By delaying the initiation of PuMA, the same experimental methodology reported here may also be used following spontaneous metastasis of tumor cells from either orthotopic or heterotopic sites.

The PuMA allows real-time assessment of metastatic progression from single cells in the lung to multi-cellular colonies. This novel experimental approach fills an important gap in the field of metastasis research by allowing real-time assessment of metastatic progression from single cells in the lung to multi-cellular colonies and beyond. The PuMA also provides an informative

unique opportunity to study the local host (i.e. stromal) determinants of metastatic progression in cancer. The description and validation of this assay provides investigators with an immediate opportunity to explore mechanisms for cancer progression at secondary sites and to optimally develop novel treatment approaches for cancer metastasis.

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Chapter 5. Evaluation of Eukaryotic Initiation Factor 4E (eIF4E) in Metastatic Osteosarcoma

ABSTRACT

The most significant problem for cancer patients is the dissemination of cancer cells and the formation of metastatic disease. Emblematic of the problem is the clinical progression seen in most patients with osteosarcoma, where metastasis to the lung is the most common cause of death. Cancer cells are believed to efficiently regulate protein translation at specific times and locations in a cell in response to changes in their environment. Within the process of protein translation the abundance and activation of the mRNA cap-binding phosphoprotein, eukaryotic initiation factor 4E (eIF4E) is considered to be both rate- and process-limiting. Based on this observation we asked if eIF4E was necessary or sufficient for metastasis in preclinical models of osteosarcoma. We overexpressed and knocked down eIF4E expression in murine and human osteosarcoma cell lines and then evaluated the consequences at various steps within the metastatic cascade in vitro and in vivo. Here, we report that suppression of eIF4E significantly delayed migration ($P < 0.001$) and reduced the number and size of colonies that formed in soft agar ($P < 0.001$). Additionally, suppression of eIF4E inhibited spontaneous pulmonary metastases. eIF4E overexpression did not change the phenotype of previously non-metastatic cells. Taken together, our data suggest eIF4E may be a necessary, but not sufficient, requirement for metastasis in osteosarcoma.

INTRODUCTION

Osteosarcoma is the most common malignant tumor of bone. The primary cause of death for these patients is the development of metastasis. Despite intensification of chemotherapy and multimodality treatments, 30% of patients with localized disease will have recurrence within 5 years, primarily to the lung. Furthermore, less than 20% of patients that present with metastatic disease will be cured. Long-term outcomes for osteosarcoma patients have not improved in over 20 years (Meyers, 2009). In order for patient outcomes to improve, we must improve our understanding of osteosarcoma metastasis.

The metastatic cascade is a complex, multistage process. Within this process, each step is subject to a wide variety of influences. The progression from a normal cell to a malignant tumor involves numerous genetic alterations. Metastasis promoters and metastasis suppressors are two broad classes of genes that contribute to the metastatic phenotype. These genes are thought to function in normal development and physiology such as cell migration, tissue invasion, and angiogenesis, but are co-opted by the cancer cell in the metastatic cascade (Khanna and Helman, 2006). Ezrin, the cytoskeleton linker protein that connects the plasma membrane to the actin cytoskeleton has been identified as a metastasis-associated gene in murine, canine, and human osteosarcoma (Khanna *et al.*, 2004). We have hypothesized and demonstrated that ezrin provides a survival advantage to metastatic cells by enabling the efficient translation of critically required proteins (personal communication, Joe Briggs). The efficiency of expression of proteins involved in cell growth regulation, proliferation or cell death may be controlled at the translational level by changes in the activity of components of the protein synthesis machinery.

There is now a growing body of evidence that suggests links between dysregulation of protein synthesis and malignant progression of cancer. To progress through the metastatic

cascade requires cooperative function of numerous proteins that facilitate invasion (e.g., matrix metalloprotease (MMPs)), survival (e.g., Bcl-2), and angiogenesis (e.g., vascular endothelial growth factor (VEGF)) (Graff and Zimmer 2003). Although expression of these proteins may be regulated at many levels by various stimuli, translation of proteins is likely regulated primarily by eukaryotic initiation factor 4E (eIF4E). A model describing a hierarchy of weakly and strongly translated proteins in cells has emerged (Baserga, 1990; Graff and Zimmer, 2003). These proteins are differentially regulated by translation. The "strongly translated" proteins are a stable set of "less regulated" proteins with short, unstructured 5' untranslated regions (UTRs) that are expressed at a basal rate with limited input from external stimuli. In contrast, "weakly translated" proteins typically have lengthy, G-C rich, highly structured 5'UTRs and are maintained as stable mRNA transcripts. These so-called weakly translated proteins are not translated unless the translational machinery is upregulated by stimuli such as growth factors or cellular stress (Bjornsti and Houghton, 2004; De Benedetti and Graff, 2004; Sonenberg and Dever, 2003). The model of weakly and strongly translated proteins suggests that under specific signals and requirements, specific proteins can be translated and delivered to specialized locations in the cell (Richter and Sonenberg, 2005). For example, when the cell is exposed to stress or growth factors, there is an increased concentration of eIF4E available to bind with the eIF4F complex creating the specifically enhanced translation initiation the weakly translated proteins require for efficient translation. The end result is disproportionately enhanced translation of weak proteins (De Benedetti and Graff, 2004). Cancer cells are believed to efficiently regulate protein translation at specific times and locations in a cell in response to changes in their environment.

eIF4E is a 25 kDa cytosolic cap-binding protein that is involved in the mRNA-ribosome

binding step of eukaryotic protein synthesis. It exists as both a free form and as part of a multiprotein complex termed eIF4F. Other subunits of the eIF4F translation initiation complex include eIF4A, a 46-50 kDa ATP-dependent polypeptide that has RNA helicase activity and a 185-220 kDa scaffolding polypeptide termed eIF4G that acts as a docking site for several proteins needed to bridge the ribosome and mRNA. eIF4G binds to both eIF4E and eIF4A (Gosselin *et al.*; Haghighat and Sonenberg, 1997) . Since eIF4E is the least abundant component of the translation initiation machinery, its abundance and activation are considered both rate- and process-limiting within translation initiation (Fan *et al.*, 2009; Pain, 1996). The function of eIF4E is to bind the 7-methyl guanosine (m7G) cap structure found on the 5' end of an mRNA and bring it to the ribosome for protein synthesis. The activity of eIF4E is modulated by phosphorylation, regulation of its transcription, and its interaction with eIF4E-binding proteins (4EBPs) (Raught and Gingras, 1999). In addition, eIF4E's activity is affected by a wide range of extracellular stimuli including growth factors, hormones, availability of nutrients, cytokines and various cellular stresses (Pain, 1996).

Increased levels or activity of eIF4E results in selectively enhanced translation of a subset of mRNAs that play a role in cell proliferation (cMYC, CDK2, cyclin D1), angiogenesis (VEGF, fibroblast growth factor 2 (FGF2)), evasion of apoptosis (Mcl-1, Bcl-2, survivin), and metabolism (MMP9, heparanase) (Hsieh and Ruggero, 2010; Konicek *et al.*, 2008; Silvera *et al.*, 2010). Under physiologic conditions, these proteins are translationally repressed but are activated in cancer. Through this translational control of cancer-associated genes, eIF4E is believed to be an important modulator of cell growth and proliferation (Sonenburg and Gingras, 1998) and is overexpressed in a number of malignancies including lymphomas, cancers of the breast, lung, head and neck, bladder, prostate, colon and rectum (De Benedetti and Graff, 2004),

esophagus (Salehi, 2006), skin (Salehi, 2007), and cervix (Matthews-Greer, 2005). While there has been a wealth of evidence in both experimental cancer models and in human cancer tissues implicating eIF4E with tumor development and progression, the majority of this work has been conducted in epithelial tumors and mouse fibroblast cells. Accordingly, we first asked about the expression and distribution of expression intensities for eIF4E in human osteosarcoma. Using immunohistochemical expression profiling of eIF4E in a human osteosarcoma TMA including pre-treatment excisional biopsies, post-treatment definitive resections, and lung metastases, our laboratory recently determined eIF4E to be similarly expressed in both primary tumors and metastatic lesions of these osteosarcoma patients (Chapter 3). In the current study we sought to define the biological role of eIF4E in the metastatic phenotype of osteosarcoma by stably overexpressing and knocking down eIF4E in poorly and highly metastatic mouse and human osteosarcoma cell lines respectively and evaluating various steps of the metastatic cascade in vitro and in vivo. We found that forced constitutive overexpression of eIF4E in both mouse (K12) and human (HOS) osteosarcoma cells was not sufficient to change the metastatic phenotype of poorly metastatic cells in vitro or in vivo whereas, suppression of eIF4E decreased cellular migration, anchorage-independent growth, and spontaneous metastasis in vivo of human osteosarcoma cells (HOS-MNNG)

MATERIALS AND METHODS

Osteosarcoma cell lines and media

High- and low-metastatic clonally related murine (K7M2 and K12, respectively) (Khanna *et al.*, 2000) and human (HOS-MNNG and HOS, respectively) (American Type Culture Collection, Rockville, MD) osteosarcoma cells lines were maintained in vitro using Dulbecco's Modified

Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) culture media containing 10% fetal bovine serum, L-glutamine (2 mmol), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO_2 incubator. For all in vitro and in vivo assays, cells were harvested using trypsin/EDTA (Invitrogen) from cultures at 70-95% confluence. All cell lines used were from early passages. Serum-free medium (SFM) and medium containing 5% fetal bovine serum were also used in select in vitro experiments. All cells were verified as Mycoplasma-free.

Overexpression lentiviral constructs and virus production

Constitutive stable overexpression of eIF4E was achieved by infecting poorly metastatic human (HOS) and murine (K12) osteosarcoma cell lines with a lentiviral construct, pSICO-CMV-cMyc-meIF4E (human; HOS-4E) or pSICO-Pol2-cMyc-meIF4E (murine; K12-4E) which contained full-length eIF4E sequences. Controls were HOS and K12 cells infected with the lentiviral pFUGW-CMV-IRES-eGFP (HOS-EV) or pFUGW-Pol2-IRES-eGFP (K12-EV) expression vectors lacking eIF4E cDNA sequences. These lentiviral plasmid DNAs were co-transfected with Invitrogen packaging plasmids into HEK293T cells to generate VSV-g pseudotyped lentivirus particles. These cells were refed with complete medium 24 hours post-transfection and the culture supernatant harvested 48 hours post-transfection. The supernatant was clarified by centrifugation and filtration. To perform lentiviral infections, 1 x 10^5 target cells were plated in a 6-well plate to 70% confluence and incubated overnight at 37 °C in a 5% CO_2 chamber. On the day of infection, the culture medium was replaced by the appropriately titered viral supernatant (1 ml/well) and incubated overnight at 37 °C in a 5% CO_2 chamber; afterward the viral supernatant was replaced with fresh medium. Forty-eight hours later, infected cell populations

were selected in Geneticin (G418; 400 mg/L). After 10 days of selection, overexpression levels were determined by Western blot analysis.

Lentiviral shRNAs and plasmids and virus production

Long-term, stable gene silencing was achieved using transduction-ready MISSION™ Lentiviral Particles (SIGMA, St. Louis, MO) that targeted human eIF4E. The target set consisted of five different sequences that were assessed individually (A-E) and pooled (P). All lentiviral constructs were assembled in the pLKO.1puro vector (www.sigma-aldrich.com/missionsearch). MISSION Non-Target shRNA Control Transduction particles were used as a negative control (NC). This non-targeting shRNA is produced from a non-target control plasmid and does not target any mouse or human genes. To perform lentiviral infections, 1 x 10^5 highly metastatic human osteosarcoma cells (HOS-MNNG) were plated in a 6-well plate to 70% confluence and incubated overnight at 37 °C in a 5% CO_2 chamber. On the day of infections, the culture medium was replaced by the appropriately titered viral supernatant A, B, C, D, E, P, or NC (150 μl/well) and incubated overnight at 37 °C in a 5% CO_2 chamber; the viral supernatant was then replaced with fresh medium. Forty-eight hours later, infected cell populations were selected in puromycin (0.5 μg/ml). After 14 days of selection, shRNA knockdown was determined by Western blot analysis. Lentiviral particles A (HOS-MNNG-A) and D (HOS-MNNG-D) produced the greatest knockdown (Fig. 5.1D).

Western blot analysis

Cells were lysed in 1X SDS lysis buffer. Equal amounts of protein were resolved on 4-12% or 4-20% SDS-PAGE gels, and separated proteins were transferred to nitrocellulose membranes.

Membranes were blocked with 5% milk in Tris-Buffered Saline Tween 20 (TBS-T) and then incubated with rabbit polyclonal anti-eIF4E primary antibody (Cell Signaling Technology, Danvers, MA) overnight at 4 °C. This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibodies and developed by SuperSignal West Pico detection (Thermo Scientific, Rockford, IL) and subsequent exposure to film.

Cap-binding assay

eIF4E-infected cells were plated at 2 x 10^6 in a 10-cm Petri dish and incubated at 37 °C in a humidified 5% CO_2 incubator for 24 hours. Lysates were prepared using m7GTP lysis buffer (500 μl/10 cm dish). BCA Protein quantification Assays (Thermo Scientific Pierce, Rockford, IL) were performed to determine the soluble protein concentration of each cell line. Cap-binding reactions were performed at 4 °C with sepharose-CL-4B (GE Healthcare) in wash buffer 1 [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, complete protease inhibitors, complete phosphatase inhibitors] for 1 hour. Binding reactions were performed at 4 °C using m7GTP-sepharose or sepharose-CL-4B (negative control) in wash buffer 1 for 2 hours. Sepharose beads were washed 2 times with 1 ml of wash buffer 1 and one time with wash buffer 2 [20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 complete Mini, EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN), 1 mL 100X Halt™ phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL)] then resuspended in 2X SDS buffer for SDS-PAGE. Antibodies to eIF4E (Cell Signaling Technology) and c-Myc tag (Sigma) were used for Western blotting.

Wound healing assay (2D motility)

Cells were seeded onto 6-well tissue culture plates and incubated in complete DMEM at 37 °C in a humidified 5% CO_2 incubator. Once cells reached 95% confluence, the cell monolayer was scratched with a pipette tip (either a P1000 or P200) to generate a wound. The remaining cells were removed by washing with PBS and fresh complete medium to remove cell debris and a permanent black marker was used to identify the region of the "scratch" where images would be captured. Phase contrast images were captured using a Leica DMIRB inverted microscope at time points 0, 6, 12, and 24 hours. The extent of wound healing was determined by the distance (motility distance) traversed by cells migrating into the "wounded" area. The migration of the cells at the wound front image was captured, and the motility distance was measured in three independent wound sites per group. All experiments were performed in duplicate and repeated a minimum of three times.

Cell proliferation assay

One hundred microliters of cell cultures were seeded (2500 cells/well) into 96-well plates and cultured at 37 °C in a humidified 5% CO_2 incubator for 4 hours. Cell Counting Kit-8 (CCK-8; DOJINDO Molecular Technologies, Inc., Rockville, MD) was used to evaluate cell proliferation. The CCK-8 assay was performed daily over a 4-day time course. Once a day, 10 μl of CCK-8 reagent was added to cell culture and incubated for 2-3 hours. The plates were then read on a microplate reader using the reference wavelength of 450 nm. Six duplicates were done to determine each data point.

Soft agar assay for colony formation

Cell lines were plated at 250 or 2000 cells per well and mixed with a 0.3% agarose solution in DMEM containing 10% FBS and layered on top of a 0.6% agarose base layer in 24-well tissue culture plates. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 3-4 weeks and colonies were counted once a week. The results were reported as the mean number of colonies observed in 6-8 wells. All experiments were done in triplicate with two independent experiments.

Cell migration assay

In vitro tumor cell migration was assessed using a 24-Multiwell Insert System (HTS FluoroBlok, BD Biosciences) containing an 8 μm pore size PET (polyethylene terephthalate) membrane. Briefly, 0.5 ml of tumor cells (5 x 10^4 cells/ml) resuspended in serum-free DMEM was added to the upper chamber (in quadruplicate). DMEM medium containing 10% FBS was added to the lower chamber. The cells were incubated for 18 hours at 37 °C in a humidified 5% CO_2 incubator. Migrated cells were then stained with 5 mM Calcein AM (Molecular Probe) in HBSS buffer for 1 hour at 37 °C. To quantify tumor cell migration, fluorescently labeled cells were detected at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a Wallace Victor 3 microplate reader (Perkin-Elmer, Wellesley, MA). Migration experiments were repeated at least three times.

Murine experimental metastasis assays

All animal studies, including maintenance and determination of experimental endpoints were performed under approval of the National Cancer Institute, Animal Care and Use Committee. To

examine experimental lung metastasis, 1 x 10^6 cells/ 0.1 ml of HBSS were injected directly into the lateral tail vein of 6- to 8-week-old female SCID beige (HOS or HOS-MNNG) or BALB/c (K12) mice respectively. Metastatic disease progression was monitored every other week in mice injected with overexpression cells. Metastatic disease progression was monitored twice a week in mice injected with knockdown cells. K12 and HOS overexpression mouse models were terminated at 24 weeks. HOS-MNNG knockdown models were terminated at 5 weeks. In all mice, the lungs were harvested and processed for histochemical analysis.

Murine spontaneous metastasis assays

Primary tumor growth was examined by orthotopic paraosseous injection of K12 and HOS overexpression cells (2 x 10^6 cells/ 0.1 ml of HBSS) or HOS-MNNG knockdown cells (2 x 10^6 cells/ 0.1 ml of HBSS) into the left caudal gastrocnemius of 6- to 8-week-old female BALB/c or SCID beige mice, respectively, as described previously (Khanna *et al.*, 2000). Serial primary tumor volumes were measured during the course of the experiment and tumor volume was calculated by the following formula: tumor volume (mm^3) = $\pi/6$ x D x d^2, where D represents the largest cross sectional diameter (mm) of the tumor and d the cross sectional diameter (mm) at right angles to D. Overexpression (K12 and HOS) and knockdown (HOS-MNNG) model mice bearing tumors approximately 15-16 mm in size underwent surgery to remove the left hind limb (amputation). The tumors were examined histologically. Overexpression model mice were sacrificed for examination of spontaneous lung metastases at 24 weeks post-surgery. For knockdown models, mice were sacrificed for examination of spontaneous lung metastases 11 weeks post-surgery. In all mice the lungs were harvested and processed for histochemical evaluation.

Histopathology

Primary orthotopic tumors and lungs of all mice were harvested and fixed with 10% formalin for 24 hours and then transferred to 80% ethanol. Whole tissue samples were submitted to Histoserv (Germantown, MD) for processing. Histologic analyses were done using H&E staining. Serial lung sections were analyzed under light microscopy for the presence of metastases.

Statistical analyses

Differences among means were analyzed using independent t-tests. Survival curves were drawn and evaluated by Kaplan-Meier and log-rank methods using GraphPad Prism v4.0 (GraphPad Software, Inc., La Jolla, CA) and Microsoft Excel (Microsoft, Redmond, WA) software. Statistical significance was defined as a *P*-value of less than 0.05.

RESULTS

eIF4E is expressed in mouse and human osteosarcoma cell lines with differing metastatic potentials

A panel of clonally related high- and low-metastatic mouse and human osteosarcoma cell lines were analyzed for their levels of eIF4E protein expression. We observed higher levels of eIF4E in highly metastatic murine (K7M2) and human (HOS-MNNG) osteosarcoma cell lines than in their clonally related low-metastatic (K12 and HOS, respectively) partners (Fig. 5.1A), suggesting that eIF4E played a role in osteosarcoma metastasis. Next, using these cell lines, we sought to determine if eIF4E was necessary or sufficient for metastasis in osteosarcoma by examining the *in vitro* and *in vivo* consequences of eIF4E (upward and downward) modulation in low (K12 and HOS) and highly (HOS-MNNG) metastatic osteosarcoma cells. We infected the

low-metastatic (K12 and HOS) osteosarcoma cell lines with a lentiviral construct that constitutively overexpressed eIF4E (Fig. 5.1B). eIF4E is a cap-binding protein responsible for the binding of the 7-methyl guanosine (m7G) cap structure found on the 5' end of an mRNA and bringing it to the ribosome for protein synthesis. Using a cap-binding assay to assess the function of our overexpression constructs, we found that our forced overexpression of eIF4E in cells was functional (Fig. 5.1C). Conversely, stable suppression of the eIF4E gene was achieved by infecting HOS-MNNG with shRNA sequences that targeted eIF4E (Fig. 5.1D).

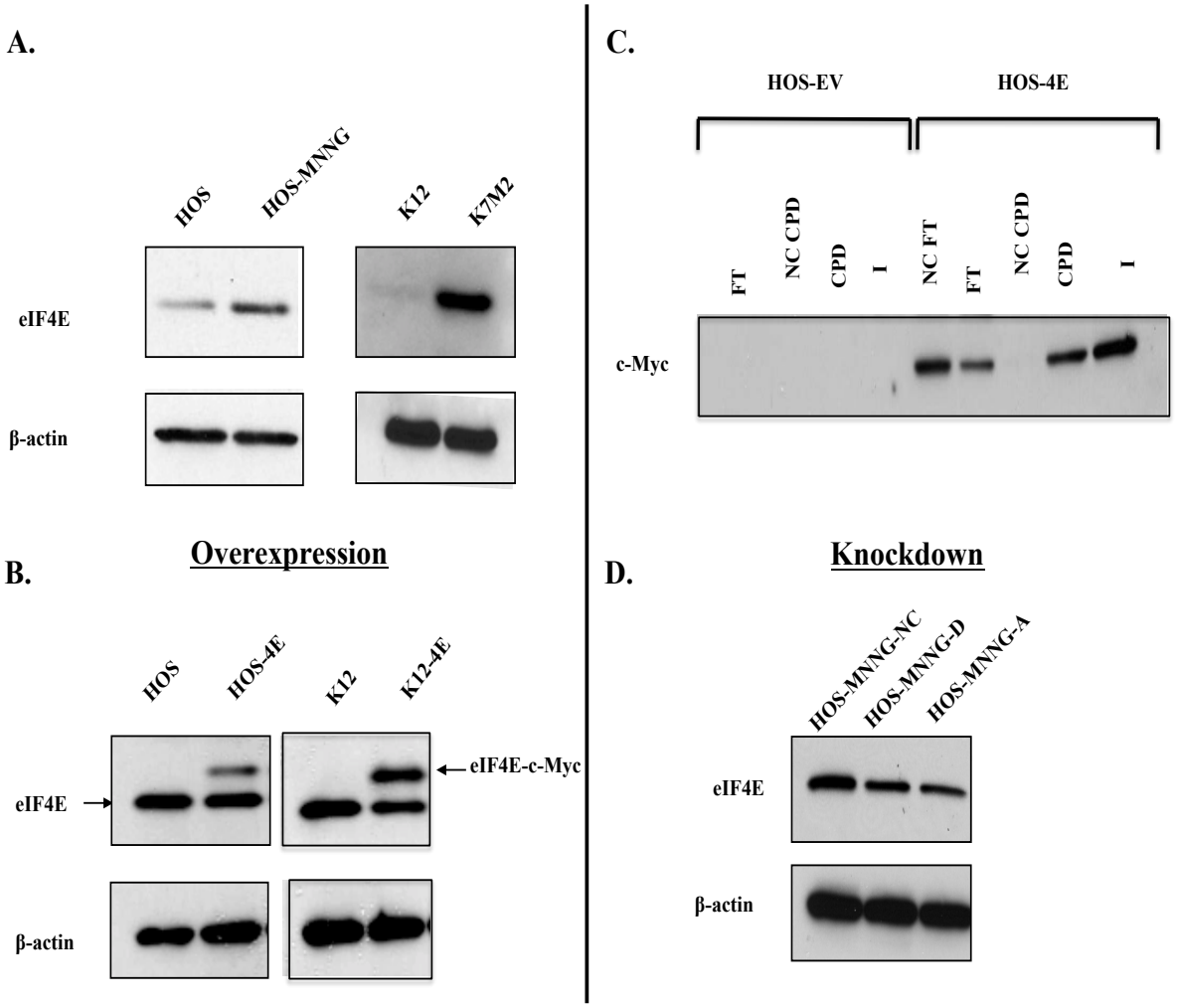

Figure 5.1. eIF4E protein is associated with metastatic potential in human and murine osteosarcoma cell lines. **(A)** Western analysis revealed higher total eIF4E in highly metastatic (HOS-MNNG and K7M2) compared to poorly metastatic (HOS and K12) cell lines. **(B)** Forced overexpression of eIF4E in poorly metastatic cell lines using a lentiviral plasmid that constitutively expressed c-Myc-tagged eIF4E. **(C)** eIF4E overexpression construct interacts with the cap complex. Epitope-tagged HOS cells overexpressing eIF4E (HOS-4E) and nonepitope-tagged empty vector control cells (HOS-EV) were incubated with 7-methyl-GTP cap beads or uncapped sepharose beads as a negative control. Immunoblots were performed with antibodies against c-Myc and actin. Input (I): proteins bound to the capped resin; Cap pull down (CPD): proteins bound to capped and uncapped resin; Negative control (NC) CPD: proteins bound to uncapped resin; Flow through (FT): unbound fraction after incubation with capped resin (C-FT) or unbound fraction after incubation with uncapped resin (NC-FT). **(D)** Highly metastatic HOS-MNNG osteosarcoma cells infected with shRNA lentiviral sequences that targeted eIF4E. Stable suppression was achieved by infecting HOS-MNNG cells with an shRNA that targeted eIF4E. Sequences A and D had the greatest knockdown. Sequences D and A are shown as the knockdown and the negative control (NC) is a non-targeted shRNA.

Overexpression of eIF4E in osteosarcoma cells does not enhance cellular proliferation, migration, or anchorage-independent growth

As eIF4E overexpression has been shown to play an important role in regulating genes that are essential for cellular growth and proliferation (Pestova and Hellen, 2000; von der Haar *et al.*, 2004), we investigated its ability to enhance these features in osteosarcoma cell lines. Overexpression did not yield a significant change in cell number in mouse (K12, $P = 0.46$) or human (HOS, $P = 0.47$) cells (Fig. 5.2A-B). Given the absence of influence of eIF4E overexpression on cell number and proliferation, we next asked if eIF4E influenced the in vitro metastatic phenotype of cells. To assess the role of eIF4E on spontaneous migratory capacity we used scratch (wound healing) assays in confluent cellular monolayers that were scratch "wounded" and allowed to "heal" for 24 hours. No increase in cell migration was seen following overexpression of eIF4E in either human or murine cell lines. In fact, the overexpression of eIF4E in HOS cells resulted in a slightly slower migratory rate compared to the empty vector control (Fig. 5.3A). Using a second assay of migration, we evaluated the role of eIF4E on serum gradient-dependent transmigration. Cells in serum-free medium were seeded on culture inserts and the ability of the cells to migrate to the underside of the insert was determined in the presence of complete DMEM with 10% FBS. Like the scratch assay, the overexpression of eIF4E did not increase migration compared to empty vector controls in either human ($P = 0.07$) or murine ($P = 0.15$) cells (Fig. 5.3B). Since the ability of cells to grow in an anchorage-independent manner is associated with increased aggressiveness, we next asked if forced overexpression would affect colony growth on soft agar. In anchorage-independent conditions (growth on soft agar), forced overexpression of eIF4E in HOS cells did not result in colony formation and there was no difference in K12 cells ($P = 0.29$) (Fig. 5.4). Collectively these data

suggest that the overexpression of a functionally active form of eIF4E was not sufficient to convert the phenotype of low metastatic cell lines in vitro.

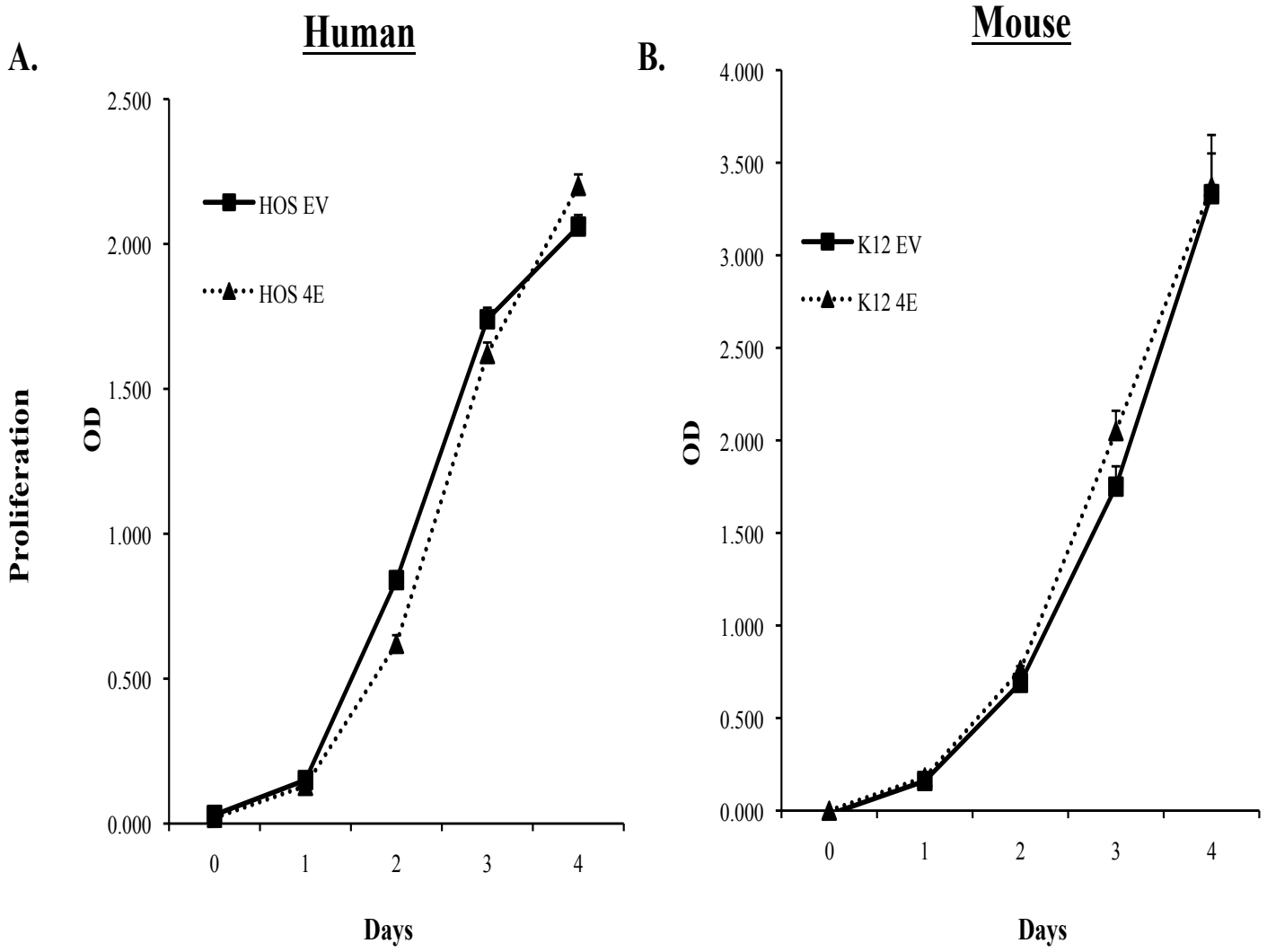

Figure 5.2. Overexpression of eIF4E in osteosarcoma cells does not influence cellular proliferation. **A-B**. Using a CCK8 proliferation assay, changes in cell growth were not observed following forced overexpression of eIF4E in human (HOS) and mouse (K12) osteosarcoma cell lines compared to empty vector (EV) controls over a 4 day time course.

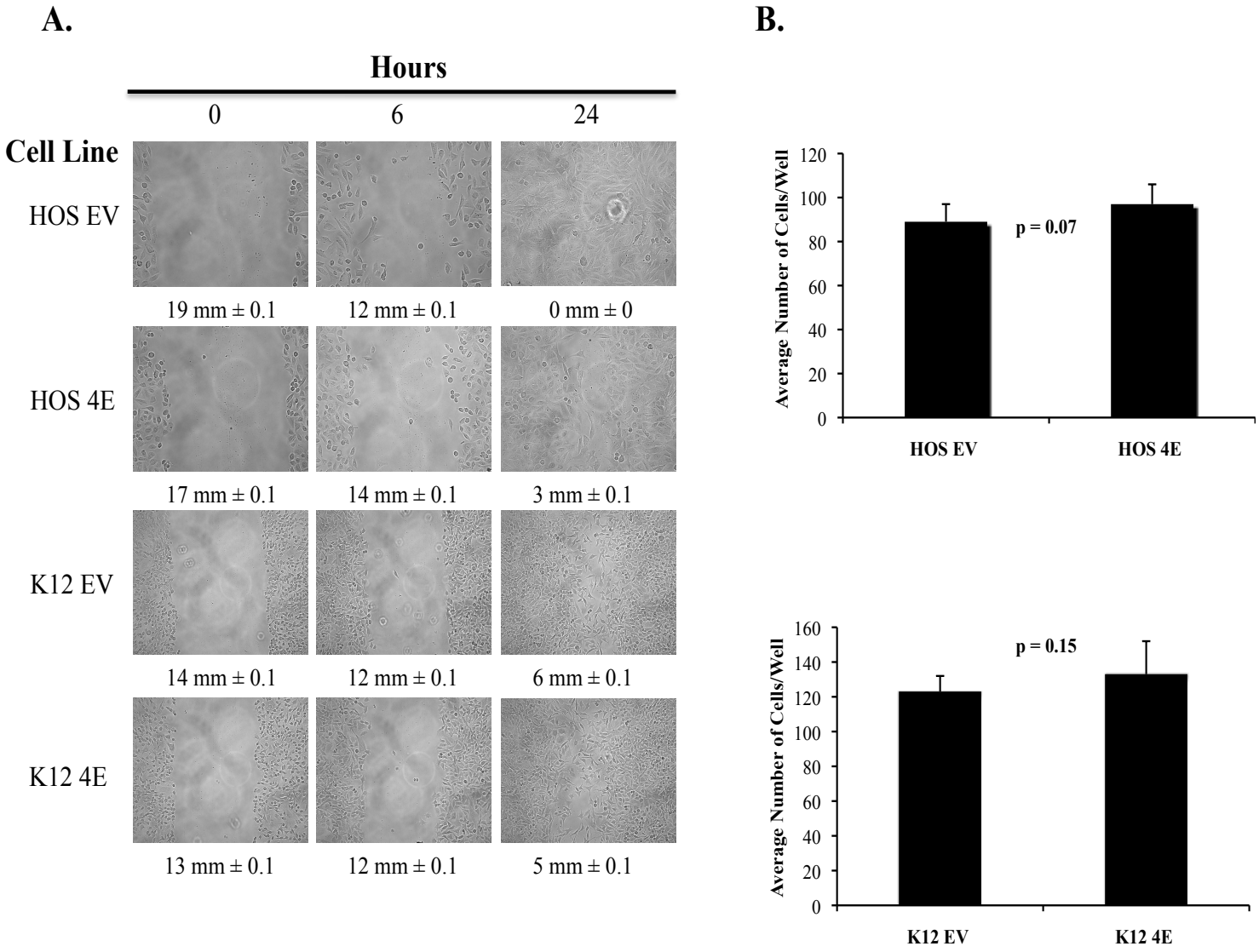

Figure 5.3. Overexpression of eIF4E in human and murine cells does not influence cell motility/migration. **(A)** Scratch assay. Cells were seeded onto 6-well culture plates. Once confluence was reached, the cellular monolayer was scratch "wounded" with a P200 pipette tip and allowed to "heal" for 24-hours. Overexpression of eIF4E in HOS and K12 cells resulted in no difference in spontaneous motility over a 24-hour time course. **(B)** Serum-gradient-dependent Transwell migration at 18h. Cells were in serum-free media were seeded onto culture inserts in 24-well plates. The ability of cells to migrate to the underside of the insert was determined in the presence of complete DMEM. There is no difference in migration in human or mouse cells when compared to controls.

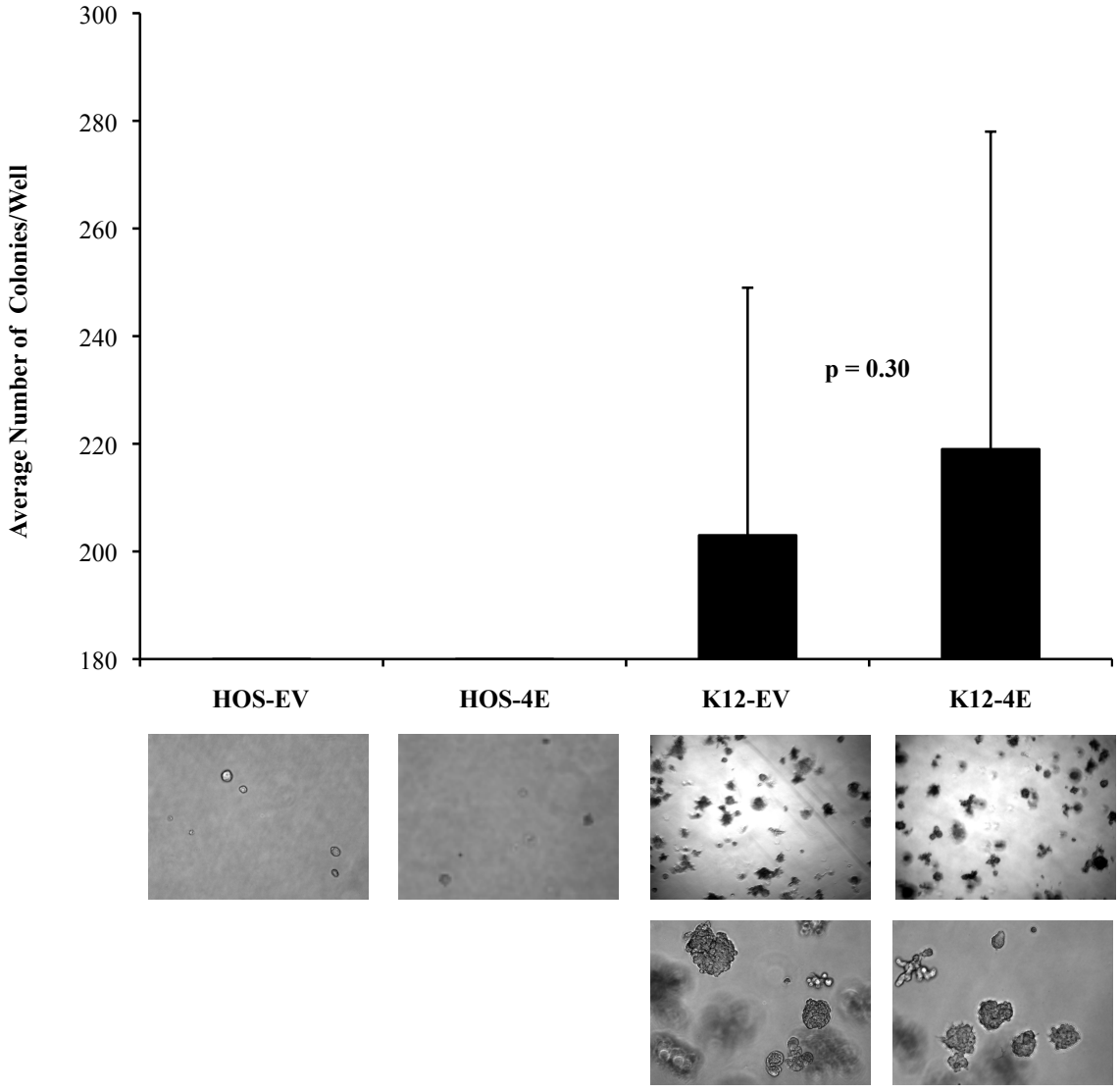

Figure 5.4. Overexpression of eIF4E in osteosarcoma cells does not influence anchorage-dependent growth. Soft Agar assay. Cells were seeded at 2000 cells per 24-well tissue culture plate and cultured in 0.3% soft agar in complete DMEM for 21 days. Overexpression of eIF4E did not result in any difference in soft agar colony formation in HOS or K12 cells. No colony formation was seen in HOS cells with or without eIF4E overexpression. All images were captured on day 21.

Overexpression of eIF4E is associated with decreased latency and faster progression of primary tumor growth, but does not enhance metastasis

Overexpression of eIF4E can promote tumor growth, as demonstrated by previous studies (Mamane *et al.*, 2004; Wendel *et al.*, 2004). The overexpression of eIF4E did not change the gross appearance of the HOS primary tumors. eIF4E overexpression resulted in a reduced time from tumor implantation to detection of tumor (tumor latency) and more rapid tumor growth (days from tumor implantation to amputation) than the empty vector control (Fig. 5.5A). Histological examination of the primary tumors revealed unencapsulated but well demarcated masses that invaded adjacent bone and skeletal muscle. The densely cellular masses contained closely packed, spindloid to elongate mesenchymal cells arranged in bundles and streams supported by a delicate fibrovascular stroma. Anisocytosis and anisokaryosis were mild to moderate and mitotic figures were high. There was multifocal hemorrhage and necrosis. Following overexpression of eIF4E in HOS cells, tumors contained small clusters of closely packed, epithelioid cells arranged in cords (Fig. 5.5B). Following resection of primary tumor-bearing limbs, mice were followed for the development of spontaneous metastasis. No pulmonary metastases were seen in mice that received empty vector control or eIF4E overexpressing HOS cells. No primary tumor development was seen following injection of control or eIF4E overexpressing murine K12 oseosarcoma cells into mice (26 weeks observation; Fig. 5.5C). To further examine the role of eIF4E on the in vivo metastatic phenotype, experimental metastasis assays were conducted with the murine and human osteosarcoma cells. Consistent with all previous data, mice given tail vein injections did not develop lung metastases 24 weeks post-innoculation

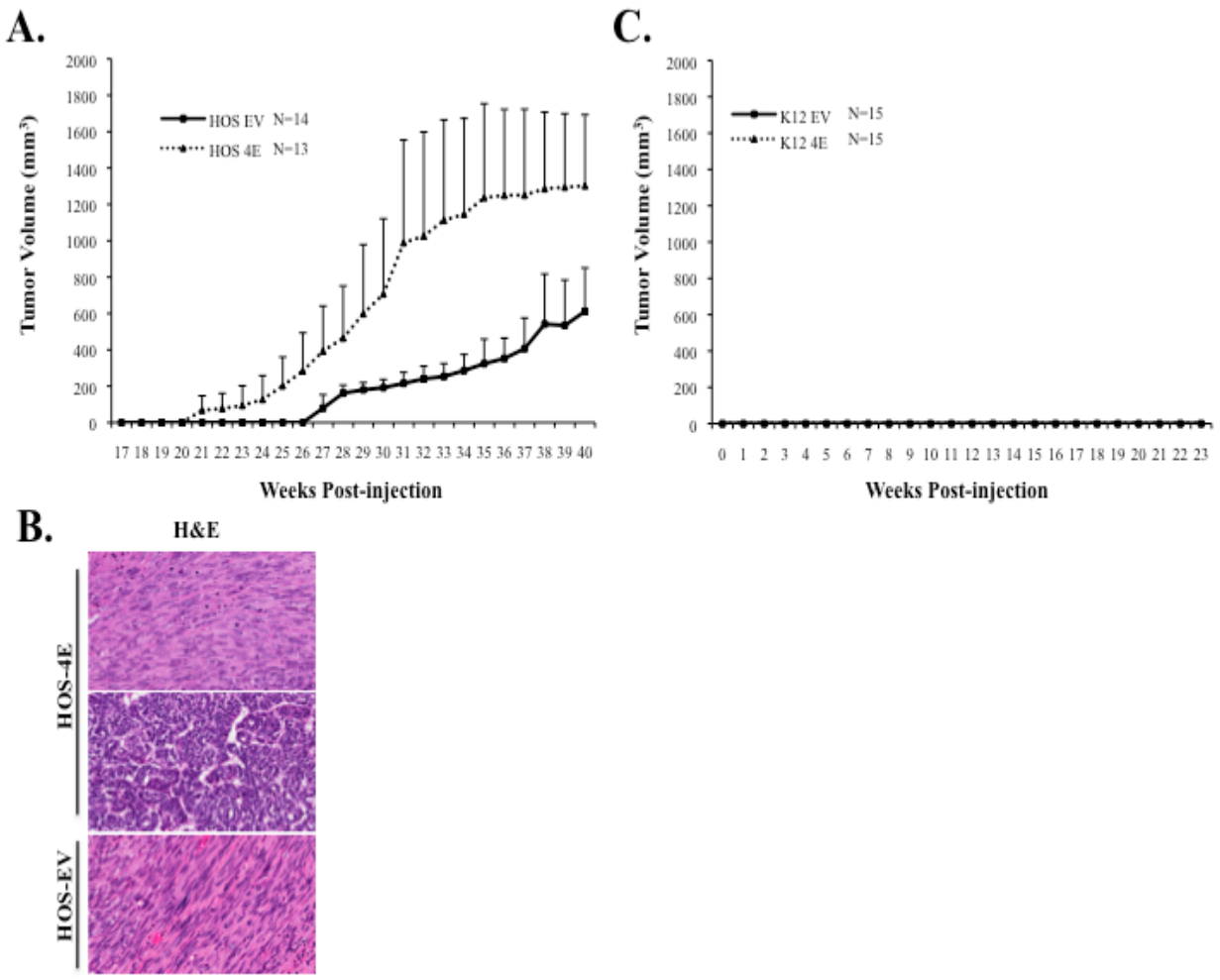

Figure 5.5. Overexpression of eIF4E in HOS cells resulted in shorter latency and more rapid growth of primary tumors over time. Primary tumor assay, HOS and K12. **(A and C)** Volume measurements of control (EV) and eIF4E overexpressing (4E) tumors grown in HOS and K12 mouse models. Shown are the averages for the indicated weeks. Overexpression of eIF4E in K12 cells is not sufficient to induce tumorigenesis in mice. **(B)** Morphologic comparison of HOS control and eIF4E overexpression primary tumors. Top and middle panels are from mice injected with HOS cells overexpressing eIF4E. HOS-4E tumors are composed primarily of spindle cells arranged in streams and bundles cells; however, these tumors also contain multiple clusters of epithelial-like cells that are often arranged in cords. The bottom panel is HOS control primary tumor. Here, the cells are composed of a monomorphic population of spindle cells.

eIF4E suppression affects tumor cell migration and anchorage-independent growth but not cell number

Inhibition of eIF4E by small molecules, antisense oligonucleotides, and small interfering RNAs can reverse the malignant phenotype in some carcinomas (Kentsis *et al.*, 2004; Moerke *et al.*, 2007; Oridate *et al.*, 2005). As such we asked whether suppression of eIF4E would influence the metastatic phenotype of osteosarcoma cells in vitro and in vivo. Suppression of eIF4E in the highly metastatic human osteosarcoma cell line, HOS-MNNG had no effect on cell number (Fig. 5.6). Cell migration was first assessed using a scratch assay for 12 hours. Cells with knockdown of eIF4E had a significant delay in migration compared to controls ($P < 0.001$) (Fig. 5.7A). Next we evaluated the role of eIF4E using a serum gradient-dependent transmigration assay. Again, the knockdown of eIF4E resulted in significantly decreased cellular migration compared to control ($P < 0.001$) (Fig. 5.7B). Finally, in anchorage-independent conditions, the suppression of eIF4E in HOS-MNNG cells did not prevent soft agar colony formation; however, the knockdown did significantly ($P < 0.001$) reduce the number and size (Fig. 5.8) of colonies compared to controls.

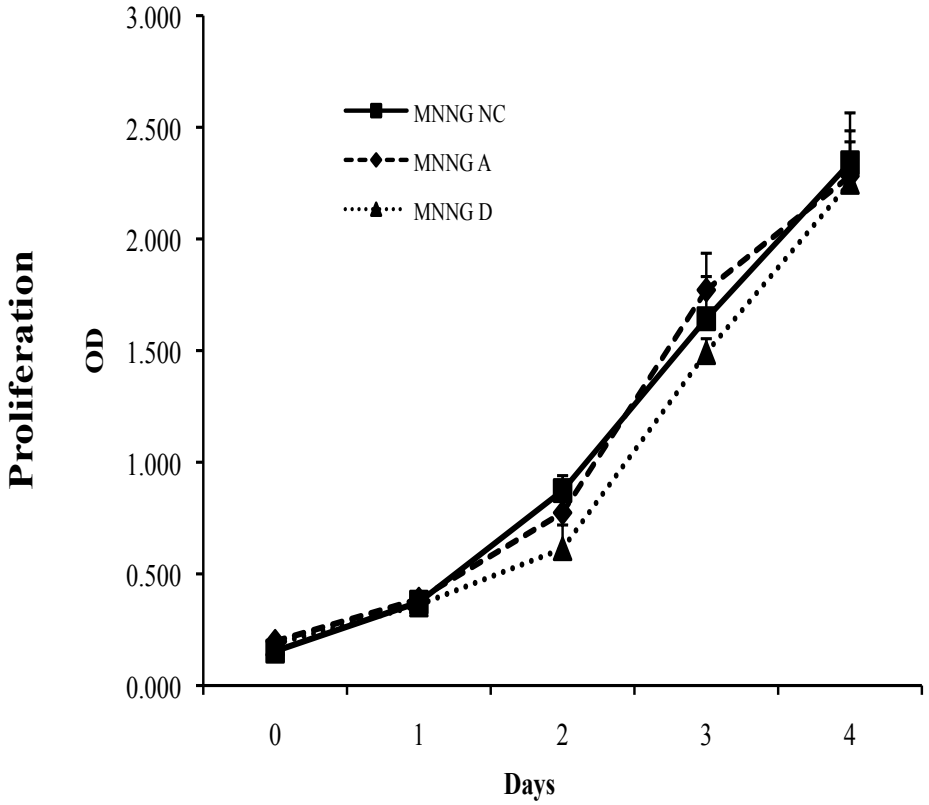

Figure 5.6. Suppression of eIF4E does not affect cell number. CCK8 Proliferation Assay. Cell growth curves demonstrated knockdown of eIF4E produced no difference in cell number in human (HOS-MNNG-A/D) cell lines compared to negative controls (HOS-MNNG-NC) over a 4 day time course.

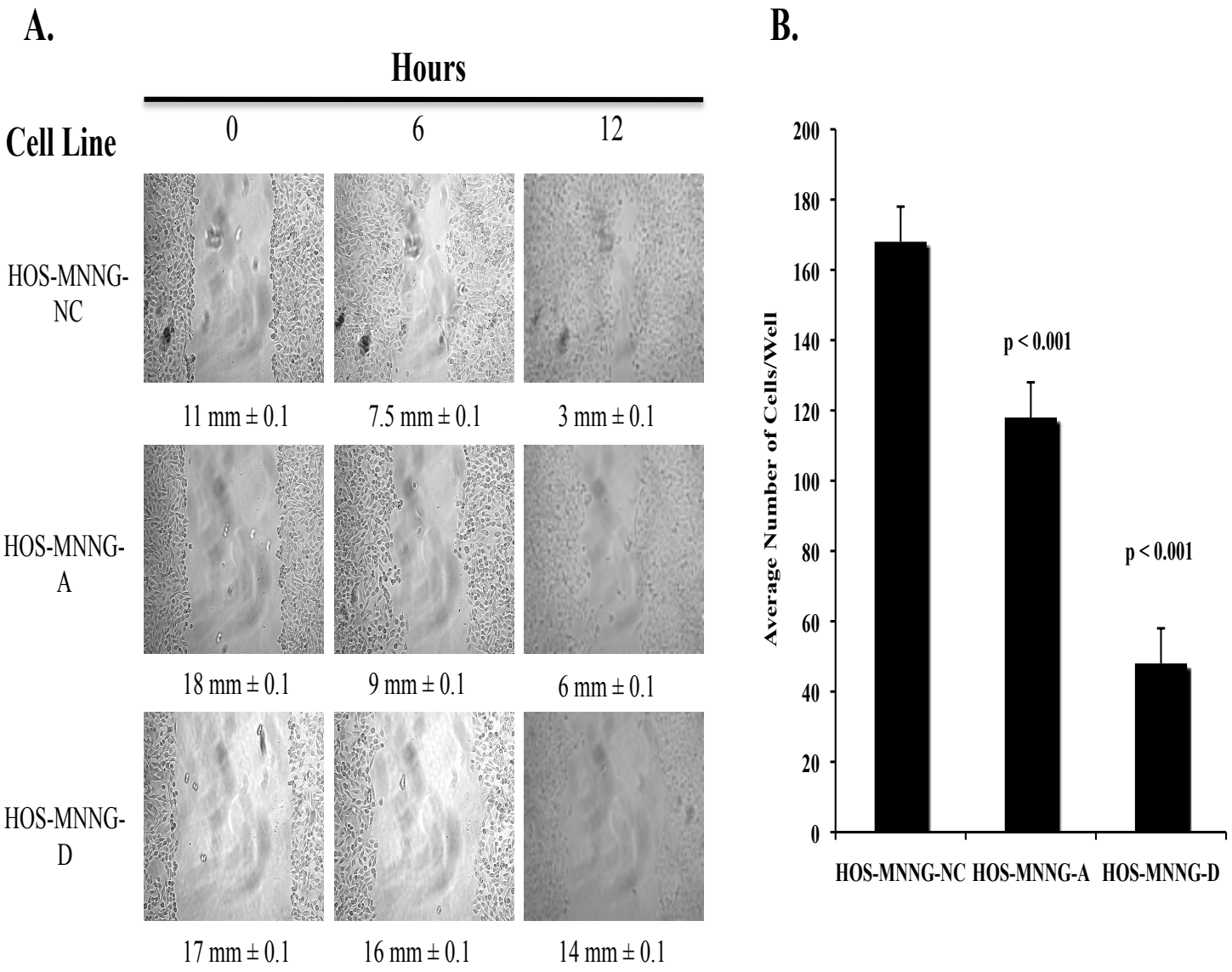

Figure 5.7. eIF4E is necessary for rapid cell motility/migration. **(A)** Scratch assay. Cells were seeded onto 6-well culture plates. Once confluence was reached, the cellular monolayer was scratch "wounded" with a P200 pipette tip and allowed to "heal" for 12-hours. Suppression of eIF4E in HOS-MNNG cells caused decreased motility when compared to negative control cells (NC) over a 12-hour time course. **(B)** Serum-gradient dependent Transwell migration at 18h. Cells in serum-free media were seeded onto culture inserts in 24-well plates. The ability of cells to migrate to the underside of the insert was determined in the presence of complete DMEM. There is decreased migration in HOS-MNNG knockdown cells when compared to controls.

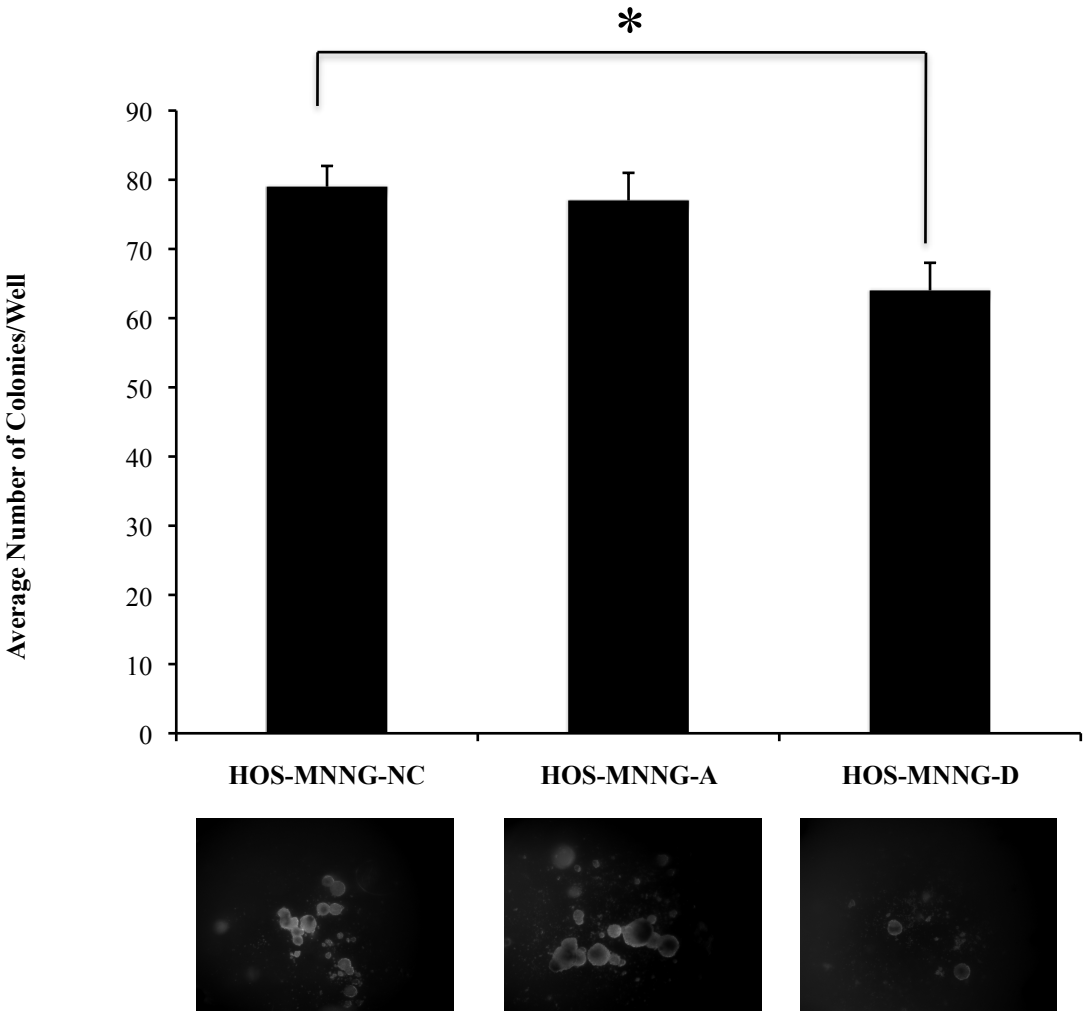

Figure 5.8. eIF4E suppression decreases anchorage-independent growth. Soft Agar assay. Cells were seeded at 250 cells per 24-well tissue culture plate and cultured in 0.3% soft agar in complete DMEM for 21 days. Suppression of eIF4E in HOS-MNNG cells reduced the number and size of colonies that formed in soft agar. All Images were captured on day 21.

Suppression of eIF4E in a mouse xenograft model reduces the number of spontaneous but not experimental pulmonary metastases

The biological behavior of the primary tumors in which eIF4E was suppressed was virtually identical to the controls. Both tumors had the same percent tumor take, latency of primary tumor growth, and a similar rate of progression (Fig. 5.9A). The gross appearance of the HOS-MNNG knockdown tumors and the negative control tumors were also similar. Histological examination of the both the knockdown and control tumors revealed unencapsulated, but well-demarcated masses that invaded adjacent bone and skeletal muscle. The densely cellular masses contained closely packed, spindloid to elongate mesenchymal cells arranged in bundles and streams supported by a delicate fibrous stroma (Fig. 5.9B). Anisocytosis and anisokaryosis were moderate and mitotic figures and apoptotic bodies were numerous. There was multifocal hemorrhage and necrosis. Following resection of tumor-bearing limbs, mice were followed for spontaneous metastasis to the lungs. Mice were sacrificed based on signs of metastasis-related morbidity. Necropsy examination confirmed lung metastasis in all mice. Following death of approximately 85% of control mice, remaining mice were sacrificed to evaluate the relative tumor burden. Grossly, pulmonary metastatic nodules were observed on the surface of both knockdown and negative control models. Grossly, knockdown mice had fewer and smaller metastatic nodules than the control mice (Fig. 5.10B). eIF4E suppression resulted in well-defined, 1-3 mm round, slightly raised, white to tan pulmonary nodules that sometimes coalesced to form larger nodules of variable size and shape. Control lungs had similar lesions but the metastatic nodules were sometimes slightly larger (1-5 mm) and formed larger coalescing masses more frequently. Histologically, pulmonary masses consisted of mesenchymal cells that look similar to the cells observed in the primary tumors for both knockdown mice and controls.

Furthermore, the suppression of eIF4E inhibited the pulmonary metastatic potential of the HOS-MNNG cells compared to the control model (Fig. 5.10A). To further examine the metastatic phenotype we used an experimental metastasis assay. The aggressive nature of this model resulted in the rapid development of metastasis in all mice (Fig. 5.11). Post mortem examination revealed that the size and distribution of the metastatic nodules was not influenced by eIF4E knockdown.

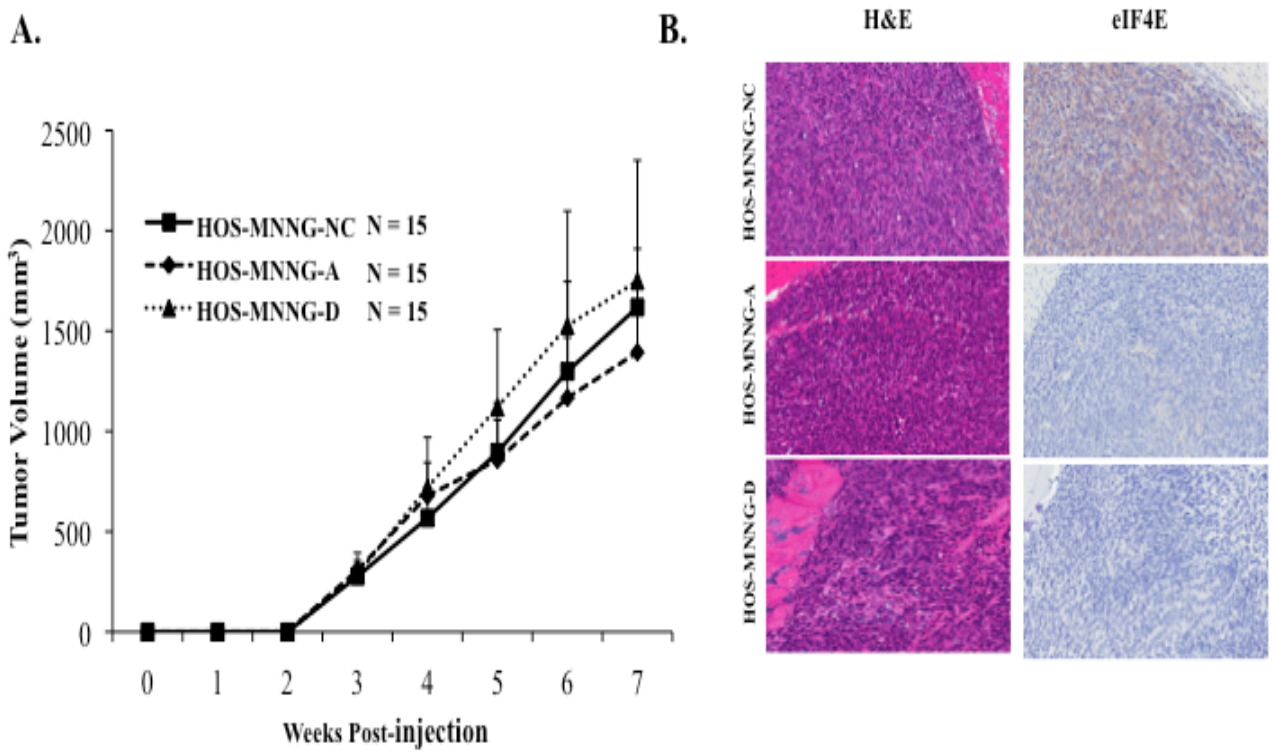

Figure 5.9. eIF4E knockdown in HOS-MNNG cells does not delay tumor formation. Primary tumor assay, HOS-MNNG. **(A)** Volume measurements of control (NC) and eIF4E knockdown (A/D) tumors grown in SCID mice. Shown are averages for indicated weeks. Suppression of eIF4E does not influence tumor formation and growth in SCID mouse xenografts. **(B)** Histologic and immunohistochemical comparison of control and eIF4E knockdown primary tumors. Histologically, both control and knockdown primary tumors were composed of monomorphic spindle cells arranged in streams and bundles. Only control primary tumors express eIF4E.

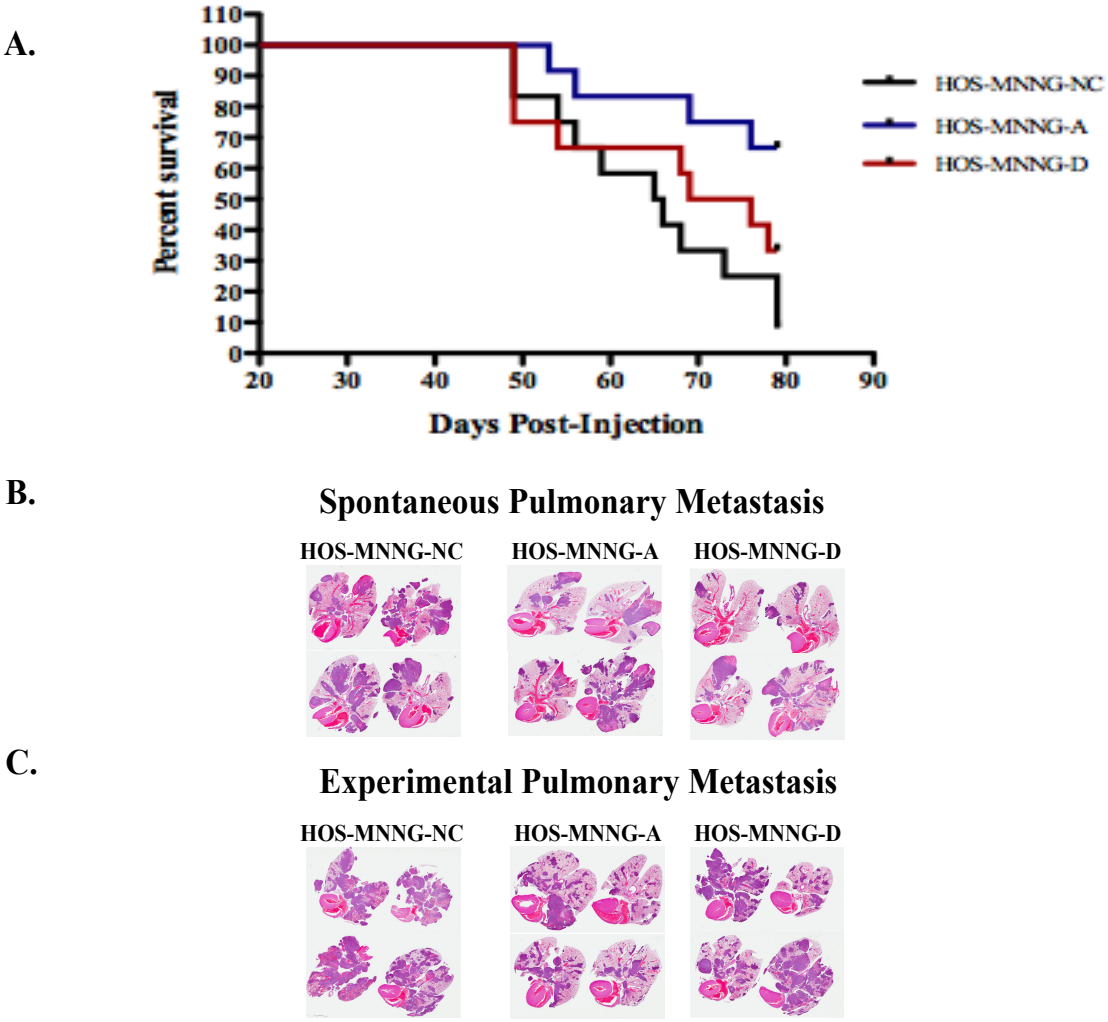

Figure 5.10. eIF4E is required for spontaneous metastasis, but not experimental metastasis. Following orthotopic paraosseous injection of control and eIF4E knockdown HOS-MNNG cells into SCID mice spontaneous pulmonary metastases developed. **(A)** Knockdown of eIF4E in HOS-MNNG cells results in a less aggressive model phenotype with a longer spontaneous pulmonary metastasis associated morbidity free interval compared to control HOS-MNNG cells. **(B)** Suppression of eIF4E in HOS-MNNG cells results in fewer spontaneous metastatic nodules compared to controls. **(C)** Injection of the same cells into the tail vein (experimental) gave rise to lung metastases without differences between the control and knockdown.

DISCUSSION

eIF4E overexpression has been associated with malignant transformation and cancer progression in a number of settings (De Benedetti and Graff, 2004). Overexpression of eIF4E is hypothesized to selectively enhance translation of metastasis-related genes (e.g. cyclin D1, VEGF, survivin, and MMP9), which are necessary for cancer cells to progress through the metastatic cascade for the successful establishment of metastases (De Benedetti and Graff, 2004; Zhou *et al.*, 2010). In this study, we explored the role of eIF4E in osteosarcoma metastasis, taking into account its known role in malignant progression in some carcinomas. We observed that clonally related mouse and human osteosarcoma cells differentially express eIF4E. Western analysis revealed higher levels of eIF4E in highly metastatic mouse (K7M2) and human (HOS-MNNG) osteosarcoma cell lines than in their clonally related low metastatic (K12 and HOS, respectively) partners. This finding provided the basis to select cells based on eIF4E protein expression levels to study the in vitro and in vivo consequences of its modulation. Thus, K12 and HOS cells were selected for overexpression studies and HOS-MNNG cells were selected for knockdown studies to further investigate the effects of enhanced and suppressed expression of eIF4E on metastatic behaviors such as tumor cell proliferation, motility, migration, anchorage-independent growth, tumor growth, and metastasis.

Cellular proliferation is important in cell cycle regulation, tumor progression, and therapeutic response. Overexpression of the eIF4E oncoprotein plays a critical role in cellular proliferation by enhancing the translation of a subset of mRNAs (cMYC, CDK2, cyclin D1) involved in important cellular processes that are implicated in oncogenesis associated with cell proliferation (Konicek *et al.*, 2008; Silvera *et al.*, 2010). In contrast to what is observed in many carcinomas, we found that modulation of eIF4E expression did not affect proliferation of

osteosarcoma cells. Suppression did not inhibit HOS-MNNG cell growth nor did forced overexpression enhance cell number in K12 and HOS cell lines.

There is a large body of experimental and clinical data documenting that carcinoma of the breast, lung, colon, prostate, and head and neck contain elevated levels of eIF4E. In addition, in animal models, eIF4E overexpression is correlated with increased number of tumors, invasion, angiogenesis, and metastasis (Crew JP, 2000; De Benedetti and Harris, 1999; Zimmer *et al.*, 2000). In this study we show that forced overexpression of eIF4E in mouse (K12) and human (HOS) osteosarcoma cells is not sufficient to change the metastatic phenotype of poorly metastatic cells to a more aggressive phenotype in vitro or in vivo. Despite the fact that overexpression of eIF4E did not change the metastatic phenotype of HOS and K12 cells, we observed that while forced overexpression of eIF4E did not enhance cell number in vitro, it was however associated with a shorter latency and more rapid progression of primary tumor growth in HOS cells. A possible explanation is that eIF4E overexpression provides a late growth advantage for HOS cells. In which case, its influence is more likely to be observed in long-term assays such as the primary tumor growth rather than short-term in vitro assays.

Tumor-cell migration is a prerequisite for invasion and metastasis. Zhou et al, showed inhibition of eIF4E by small interfering RNAs (siRNA) effectively inhibited motility and suppressed colony formation in triple-negative breast cancer cells (Zhou *et al.*, 2010). We observed that suppressing eIF4E influenced spontaneous and serum gradient-dependent migration of human osteosarcoma cells similarly. eIF4E inhibition reduced the motility/migration of highly metastatic cells. Additionally, we demonstrated in anchorage-independent conditions, suppression of eIF4E in HOS-MNNG cells resulted in colonies that were reduced in both number and size. In the in vivo knockdown model, spontaneous lung

metastases were reduced in number and size compared to the control. There was no discernible difference in experimental metastases between the knockdown and control. Tumor take, primary tumor development, and growth rate were similar in the knockdown and control mice. Again, the fact that a difference was observed in the spontaneous metastasis (long-term) assay but not the experimental or primary tumor (short-term) assays suggests that eIF4E expression provides a late advantage in growth.

In summary, forced overexpression of eIF4E alone did not change the metastatic phenotype of poorly metastatic human and murine osteosarcoma cell lines in vitro or in vivo. However, suppression of eIF4E resulted in reduced migration, anchorage-independent growth, and spontaneous lung metastases. Taken together, our results suggest that eIF4E likely plays a necessary role in the molecular strategy adopted by osteosarcoma cells to achieve metastasis, but alone is not sufficient for motility/migration, anchorage-independent growth, tumor formation, and metastasis. Still, targeting this gene may offer clinical benefits in these tumors. Our observations on eIF4E constitute, to the best of our knowledge, the first report on the biological role of eIF4E in metastatic osteosarcoma. Overall, this study provides evidence that both eIF4E-dependent and eIF4E-independent mechanisms contribute to tumorigenicity in osteosarcoma cells. Because our study is based mainly on a single, clonally related human osteosarcoma cell line, exploration of eIF4E expression in additional human osteosarcoma cell lines will be required to accurately define the role of eIF4E that we report in osteosarcoma metastasis. Currently, there is very limited information about eIF4E expression and activity in cancers of mesenchymal origin, including osteosarcoma. Assuming that eIF4E plays a similar role in osteosarcoma as it does in multiple carcinoma types, we propose that therapeutic strategies targeting eIF4E may improve the outcome for osteosarcoma patients. Current strategies designed

to target eIF4E in cancer malignancies such as breast, prostate, stomach, colon, lung, skin, and the hematopoietic system (Hsieh and Ruggero, 2010) include antisense oligonucleotides to eIF4E, RNAi or antisense RNAs that suppress eIF4E, a physical mimic of the natural ligand, suicide gene therapy, and peptide-based inhibition of eIF4E (Ko, 2009). Our results suggest targeting eIF4E in osteosarcoma may offer clinical benefits.

Acknowledgements

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Chapter 6. Conclusions and Future Directions

The development of metastases, most commonly to the lung, is the cause of death for most osteosarcoma patients. Long-term outcomes for osteosarcoma patients have not improved in over 20 years (Meyers, 2009). The primary research need in the field is to understand the biology of metastasis in osteosarcoma so as to improve outcomes for future patients. Unraveling the complexity of metastasis demands a focus on new tools, reagents, and biology in order to investigate hypotheses. Accordingly, this body of work introduces an outcome-linked human ostoesarcoma tissue microarray (new tool) and an ex vivo pulmonary metastasis assay (new reagent) that allows real-time assessment of metastatic progression. In addition, we describe for the first time, the biological role of eIF4E (new biology) in metastatic osteosarcoma. The goals of this research are to utilize these new tools and reagents to identify proteins and/or processes that define the metastatic phenotype of osteosarcoma and to use our newfound understanding of eIF4E in osteosarcoma metastasis to develop novel therapeutic strategies to prevent growth of metastases and improve treatment outcomes for patients.

Human osteosarcoma tissue microarray (TMA)

In this work, we have described a human osteosarcoma tissue microarray (TMA) that we believe will aid in the advancement of our understanding of both metastasis biology and therapeutic strategies. We used this tool to detect and validate protein biomarkers across a variety of patients. Our outcome-linked TMA was constructed to compliment other TMAs currently available and address the limitation of small sample size while providing a wider variety of sample types that characterize the disease including pre-treatment excisional biopsies,

post-treatment definitive resections, and lung metastases. Eighty-nine tissue specimens collected between 1984 and 2001 were obtained from 75 patients (35 males and 40 females) undergoing resection of osteosarcoma of the extremities, pelvis, and craniofacial bones. A variety of anatomic sites and histologic subtypes (see Table 3.1) were included. Using these tissues and associated clinical annotation, a schema of tissue cores, arranged according to the sample type (biopsy, definitive resection, or resection of distant metastasis), was developed. Final patient specimens included: 21 primary biopsies (19 patients), 48 definitive resections (47 patients), 20 metastases (14 patients), and 12 control tissues (see Fig. 3.1). As an example of our TMA's utility we evaluated the immunohistochemical expression of eIF4E in osteosarcoma tissues formatted on this array and assessed correlations between eIF4E expression, sample type, and overall survival. Our findings demonstrated relatively uniform expression of eIF4E in both primary tumors and metastatic lesions of these osteosarcoma patients (see Fig. 3.2A) and that eIF4E expression intensity was not an independent predictor of overall survival (see Fig. 3.3B).

Pulmonary metastasis assay (PuMA)

Here we described an ex vivo pulmonary metastasis assay (PuMA). We used this assay to evaluate novel therapeutics that specifically target metastatic progression and metastatic lesions in a timely manner. This closed system and sterile assay allowed real-time assessment of the metastatic progression of GFP-labeled cancer cells, from a single cell to the formation of multicellular clusters, in the mouse lung microenvironment for up to 21 days. To validate the utility of the PuMA for the study of metastatic progression, we compared previously described highly metastatic versus poorly metastatic clonally related human (Rhim *et al.*, 1977) and murine (Khanna *et al.*, 2000) osteosarcoma cell lines. This model faithfully predicted high- and low-

metastatic phenotypes of human and mouse cancer cell lines (see Fig. 4.4A-D). The clonally related variants with greater metastatic propensity in vivo were also associated with greater metastatic phenotype in the PuMA. The PuMA fills and unmet need in the study of metastasis biology and therapy by allowing the real-time assessment of metastatic progression in a relevant tumor microenvironment. Furthermore, it is a useful tool for translational drug development as it allows for assessment of multiple drug doses, exposure times, and schedules.

eIF4E and Osteosarcoma Metastasis

eIF4E overexpression has been associated with malignant transformation and cancer progression in a number of settings (De Benedetti and Graff, 2004). Overexpression of eIF4E is hypothesized to selectively enhance translation of metastasis-related genes (e.g. cyclin D1, VEGF, survivin, and MMP9), which are necessary for cancer cells to progress through the metastatic cascade for the successful establishment of metastases (De Benedetti and Graff, 2004; Zhou *et al.*, 2010). In the current study we sought to define the biological role of eIF4E in the metastatic phenotype of osteosarcoma, by stably overexpressing and knocking down eIF4E in poorly and highly metastatic mouse and human osteosarcoma cell lines respectively, and evaluating various steps of the metastatic cascade *in vitro* and *in vivo*. We found that suppression of eIF4E decreased cellular migration, anchorage-independent growth, and metastasis *in vivo* of human osteosarcoma cells (HOS-MNNG) whereas, forced constitutive overexpression of eIF4E in both mouse (K12) and human (HOS) osteosarcoma cells did not enhance cellular migration, anchorage-independent growth, proliferation, or metastasis. Collectively, our data suggest that eIF4E is potentially necessary for metastasis in osteosarcoma as inhibition of eIF4E was effective in suppressing the metastatic phenotype in highly metastatic human osteosarcoma cells

(HOS-MNNG). Conversely, and not surprisingly, forced overexpression alone was not sufficient for poorly metastatic mouse and human osteosarcoma cell lines to achieve a metastatic phenotype in vitro or in vivo.

Future Directions

While the expression of eIF4E in osteosarcoma cells and tissues along with evidence linking its inhibition with a suppressed metastatic phenotype are encouraging, our study is based mainly on a single, clonally related human and mouse osteosarcoma cell line. Therefore, it is necessary to explore eIF4E expression in additional human osteosarcoma cell lines in order to more accurately define the role of eIF4E in osteosarcoma metastasis. Presently, there is very limited information about eIF4E expression and activity in cancers of mesenchymal origin, including osteosarcoma. It is possible that through our work we may have identified a valuable new potential target for treatment of osteosarcoma. Assuming that eIF4E plays a similar role in osteosarcoma as it does in multiple carcinoma types, we believe that therapeutic strategies targeting eIF4E may improve the outcome for osteosarcoma patients. Current strategies designed to target eIF4E in various carcinomas and the hematopoietic system include antisense oligonucleotides to eIF4E, RNAi or antisense RNAs that suppress eIF4E, a physical mimic of the natural ligand, suicide gene therapy, and peptide-based inhibition of eIF4E (Hsieh and Ruggero, 2010; Ko, 2009). Given that progression through the various steps of the metastatic cascade are likely to be similar in many cancers, an important next step would be to assess agents that may already be in development or in clinical use for carcinomas in preclinical and clinical studies in osteosarcoma. The PuMA could be used to assist in the assessment of novel therapeutics, evaluating multiple drug doses, exposure times, and schedules. Our data support the concept of

evaluating these new agents in preclinical models of sarcoma as well as in dogs with naturally occurring osteosarcoma.

To date, significant information has been accumulated regarding targeting eIF4E in carcinomas however the potential of targeting eIF4E in osteosarcoma has yet to be realized. As such, control of protein synthesis has emerged as a new research area with significant potential for developing novel therapeutic approaches in the treatment of osteosarcoma. Protein synthesis is tightly regulated at the level of translation initiation. Our over-riding hypothesis suggests that eIF4E expression will define the extent to which specific "weakly" translated (or metastasis-associated) mRNA transcripts are translated. Cells with an enabled translational machinery will be able to respond to the dynamic events associated with moving through the metastatic cascade by expressing these needed proteins on time at the appropriate locations within a cell. We hypothesize that this enabled ability to express proteins in a dynamic environment will contribute to the metastatic propensity of a cell. Identifying potential metastasis-related mRNAs that are differentially regulated following eIF4E overexpression or suppression in osteosarcoma would be an important and potentially clinically relevant question in this area of research. Western blot analysis of previously described "weakly" translated proteins likely to be linked to metastatic function (see Table 2.1) would generate a list of proteins differentially expressed in osteosarcoma cells. From here, quantitative PCR could be used to examine the proteins at the transcriptional level to confirm that any differences are, in fact, the result of translational regulation of their expression. In addition, a non-candidate approach, polysome expression microarray techniques, based on identification of mRNA transcripts that are expressed with multi-unit ribosomes (so called heavy polysomes) would allow the identification of unique putative "weakly" mRNAs that are differentially expressed following eIF4E modulation that are

destined for translation by virtue of their association with multi-unit ribosomes. Finally, this improved understanding and discovery of new targets involved in translation will facilitate the use of novel inhibitors of eIF4E and translation initiation currently under development in our laboratory and elsewhere to be used in the treatment of osteosarcoma.

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